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         Exposure and Stress

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13. ABSTRACT (Maximum 200 Words)
Parkinson's disease (PD) is caused by deterioration of the dopamine (DA) nigrostriatal system. Loss of DA can be induced experimentally by neurotoxic lesion of DA neurotransmitter producing neurons in the substantia nigra, or through lesioning nigrostriatal DA terminationsin the striatum with subsequent degeneration of their cell bodies in the substantia nigra. We have characterized a new animal model of preclinical PD. Experimental PD has been induced by unilateral, inranigral infusions of the neurotoxin malonate or 6-hydroxydopamine to produce partial loss of striatal DA. The animals were assessed weekly for forelimb use asymmetries to obtain a behavioral index of the striatal DA imbalance. The striata were examined morphologically and compared to the intact, contralateral side. Striatal DA terminal losses were determined by evaluation of expression of tyrosine hydroxylase. Differntial changes in postsynaptic striatal DA receptor expression, and components of the intrinsic (mitochondrial) programmed cell death cascade occurred at 4 weeks after neurotoxin exposure. Effects of a secondary stress event on further exacerbation of striatal changes after the neurotoxin lesion are being examined. Neurochemical analysis of residual striatal DA following neurotoxin and secondary stressor exposure was performed in parallel, using HPLC of DA and its metabolites. Some tissues await evaluation.

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Introduction

This document covers research performed 01 Sep 99 through 31 Jan 04. The research established and characterized a rat model of early-stage Parkinson's disease (PD) to examine striatal elements that may be particularly susceptible to initial depletion of dopamine (DA) in the nigrostriatal pathway. Two neurotoxins were used in these investigations; 6-hydroxydopamine (OHDA) and malonate. Two different regions of the nigrostriatal pathway were targeted; 6-OHDA was infused into the DA terminal field of the striatum, while malonate was placed into the DA cell bodies in the substantia nigra. HPLC assessed striatal DA levels at 4 weeks to establish the appropriate dose to achieve ~50% of control (range of 35-75% loss detected). This partial nigrostriatal DA depletion could be monitored behaviorally using limb-use asymmetry measurements (Tillerson et al, 2001). Limb-use asymmetry does not require pre-training or shaping the rats to a criterion. This test monitored subsequent losses in striatal DA following secondary stress events, induced by brief TMT exposures (2 hours/week for 4-5 weeks, 30 µl volatilized). TMT is the active component in fox feces, and its use provided a species-relevant natural stressor that also did not necessitate pre-training the rats. Morphological evaluation of the striatum at the 4-week time period showed substantial elevations in protein staining for the D2 subtype of DA receptor and the second messenger cyclic AMP in the DA depleted striatum compared to intact. These alterations were not present in sham lesioned rats. Striatal DA loss was determined using staining for tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of DA. Following discussions with Dr. JT Greenamyre (Emory University) and Dr. RE Burke (Columbia University), indicators of striatal mitochondrial insufficiency were examined. These studies showed clear signs that neurons in the DA depleted striatum are targeted at 4-weeks but not 1 week after nigrostriatal interruption, responding with elevations in enzymes associated with induction of the intrinsic (mitochondrial) programmed cell death pathway. These changes were isolated further to phenotypically identified striatal projection neurons (Nisenbaum et al, 1996) using double-labeled immunofluorescence, and were confirmed by western analyses. The results demonstrated that imbalances in cellular homeostatic indicators predominated in the striatopallidal projection system. Cell counts determined that there was no loss in neuron density after the DA depletion. Taken together the findings suggested that activation of the intrinsic programmed cell death pathway does not initiate apoptosis in striatopallidal neurons early in PD but may predict the presence of transient molecular events as a response to changes in the neurochemical milieu of the striatum.

Body

01 year, 9/99 start through 6/00 *(The PI was on sabbatical leave at UCLA for the 1999-00 academic year. These arrangements predated the submission of the DoD grant, and thus could not be changed to accommodate the funding. The PI was refused approval from UCLA to perform animal experiments on the campus. The co-PI and a graduate student performed the animal work, and the PI advised and analyzed data at UCLA during this 9 month period).*

Task 1: *Commence bilateral striatal 6-OHDA and substantia nigra malonate infusions into young adult, male rats using various infusion doses of the toxins to establish the concentration necessary to achieve 50% loss of striatal DA.* Infusions were restricted to 6-OHDA, since the animals needed supplemental diets and increased husbandry to flourish following the bilateral insult. The 6-OHDA in ascorbate-saline was infused into the striatum in four sites per rat (2 per side). Eight animals were subjected to this regimen, using two different doses of 6-OHDA (12.5

μgm/4 μl and 6.25 μgm/4 μl). The results demonstrated that the lower dose of 6-OHDA would yield an approximately 50% loss of striatal DA at 4 weeks, determined by HPLC analyses.

Task 2: *Baseline behavioral assessment using two paradigms, skilled paw reaching and elevated bridge traverse to examine balance, posture and dexterity after the neurotoxic lesion.* The staircase apparatus (Montoya et al, 1990; Whishaw et al, 1997) tested skilled forepaw reaching for reward pellets following 12 hours of food deprivation. Training included as many as 16 sessions of 15 minutes duration each. Rats needed shaping to acquire this activity using bait on the first 4 stairwells. Since there are six wells on each side, loaded with two reward pellets, a "perfect" score in the test session would be 24. All animals showed a learning curve typical for the acquisition of a new motor skill. Successful pellet grasp and consumption (hit), attempted grasp (miss), and no attempt (no try) were documented for each stair well for each session in which the rat was tested. Records were made of the preferred paw used to reach for the reward to assess handedness of the animals. The balance, motor sequencing and duration of the behavior also were recorded. The elevated bridge behavioral paradigm was not examined as components of balance and posture could be determined in the skilled forepaw reaching task.

Baseline Performance
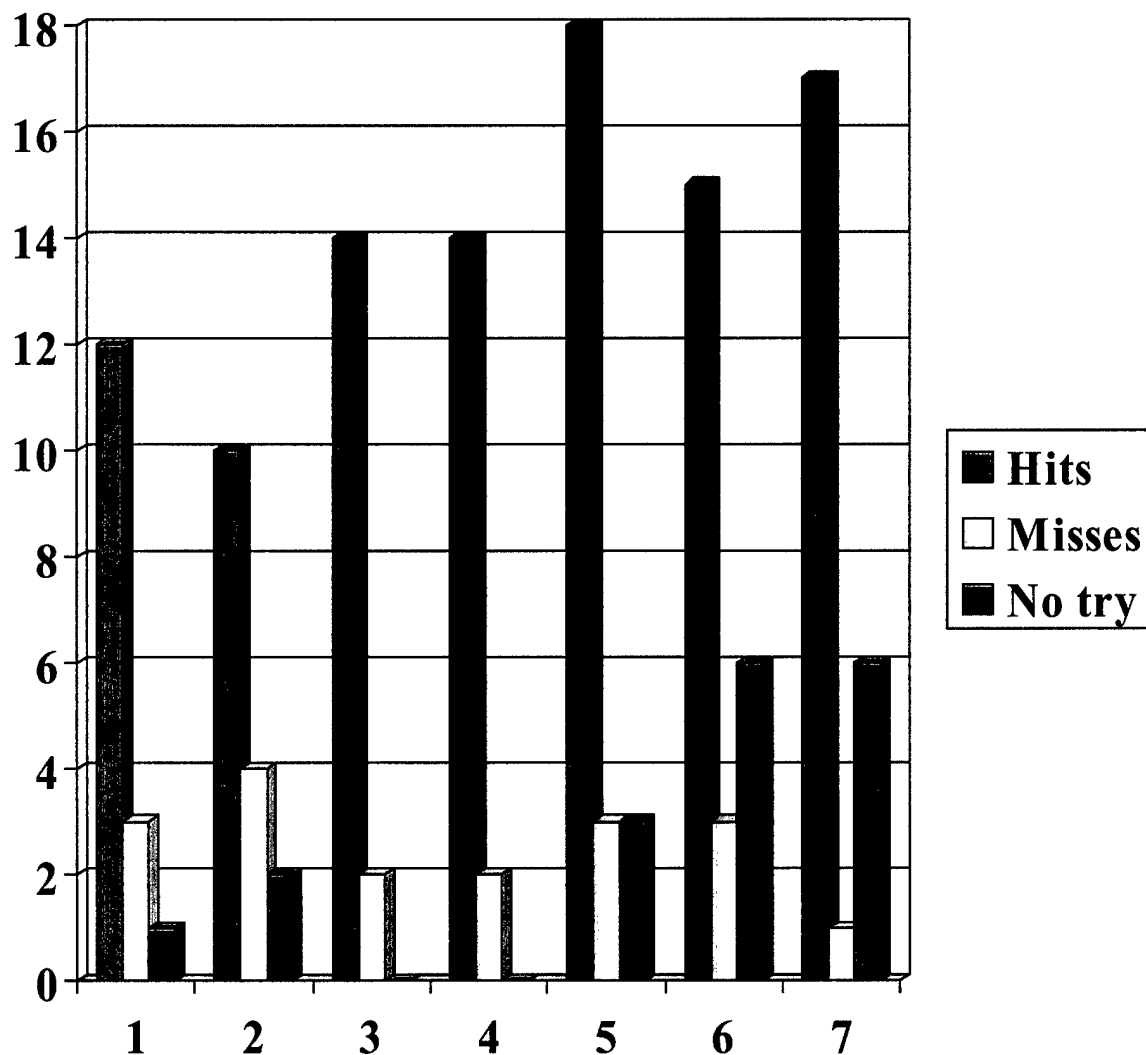
Task 3: *Behaviorally assess sham (N = 3) and 6-OHDA bilaterally lesioned (N = 9) animals within 24 hours of the surgery, then extract brains for subsequent biochemical and morphological evaluation.* The short-term behavioral analysis could not be performed due to the status of the neurotoxin treated animals. Thus, rats were evaluated one week after the neurotoxin infusion to ensure their recovery. This was due to our concern that the animals should not be food deprived because of their tender condition in the first week following the bilateral striatal DA lesions. The behavioral results were not as consistent in the lesioned animals at 1 week as the sham group. A learning curve was detected, but rats were not as motivated to obtain the reward pellet. Animals needed more time to consume the pellet, and as a consequence the 15-minute test session was too short for adequate performance evaluation in the lesioned animals. Postural impairments included an inability to position the body to reach appropriately for the reward pellet. Motor sequencing was impaired and chewing was abnormal. These were evaluated by video taped sessions. Rats were killed 24 hours after the last behavioral session and the brains frozen.

Performance after Bilateral 6-OHDA
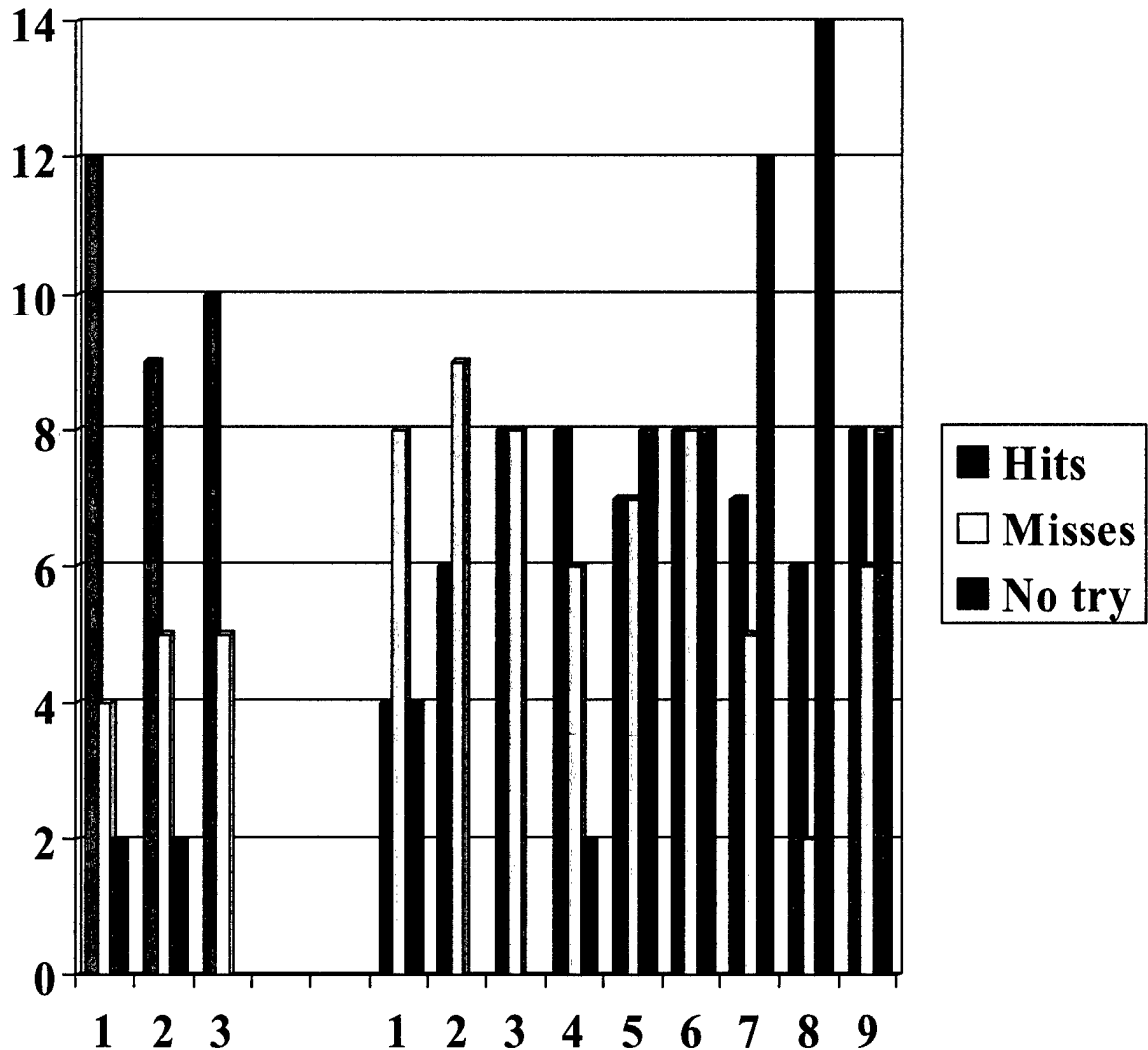
Task 4: *HPLC assessment of DA and its metabolites.* A series of animals were lesioned at the two doses of 6-OHDA and sent to our collaborator, Dr. John Elsworth (Yale University) for HPLC analysis. These results verified that the lower dosage of 6-OHDA yielded ~ 50% depletion of striatal DA at 4 weeks.

Task 5: *Morphological evaluation of striatal DA receptor subtypes, and DA.* Brains were examined and showed consistent elevations in striatal D2 DA receptor protein staining following partial DA depletion compared to naïve brains, processed concurrently. The other DA receptor subtypes (D1, D3, D4, and D5) did not have dependable outcomes. The loss of striatal DA was evaluated using immunofluorescent detection of TH. Rats that showed no loss in TH did not demonstrate changes in D2 DA receptor expression. Only rats with ~50% TH depletion from sham or naïve animals were examined morphologically.

02 year 7/00 start through 6/01 *(The SOW was modified – appendix materials – because of our concerns with the health of the rats and reliability of the data. New experiments were initiated using unilateral, partial 6-OHDA intrastriatal infusions.)*

Task 1: *Commence unilateral striatal 6-OHDA infusions.* We used 6 µgm in 2 µl ascorbate-saline to produce 50% loss of striatal DA. HPLC was performed at 1-week and 4-weeks and confirmed that this dose would achieve our criterion at the 4-week time point. ANOVA and Fisher posthoc least squared difference showed significant differences in DA levels of the 6-OHDA treated striatum ($p<0.0001$) compared to the control side at both time points (N = 9). Striatal DA turnover was elevated significantly at 1 week ($p< 0.004$) and 4 weeks ($p < 0.03$).

Task 2: *Behavioral indications of striatal DA imbalance.* The severity of nigrostriatal DA damage was evaluated using a sensitive behavioral task that examined limb-use asymmetries (Schallert and Tillerson, 1999; Tillerson et al, 2001). This task did not require pre-training the rats. The procedure for scoring and analysis is included in the appended manuscript in review at *Experimental Neurology* (Ariano et al, 2004). Results of limb-use asymmetry in rats with partial, unilateral DA depletions are depicted in figure 1 of the manuscript. This manuscript reports on only one neurotoxin, 6-OHDA infused into the substantia nigra (SN), however findings were equivalent for intrastriatal 6-OHDA.

Task 3: *Evaluate the morphological staining for the five DA receptor subtypes within the unilateral intrastriatal 6-OHDA lesioned rats, at time points following the infusion at 1 week (N = 4 rats) and 4 weeks (N = 9 rats).* We used TH immunofluorescence to evaluate striatal DA depletion. The TH staining in paired images from equivalent striatal regions in each control and DA depleted striata showed DA terminals throughout the parenchyma of the intact striatum, but this pattern was considerably attenuated in the 6-OHDA infused striatum. At 1 week, the striatal DA depletion was 54.7% (range of 30-85%) of the control side. For the 4-week sample, the TH staining was 48.8% loss on the depleted side (range 40-75%). A typical reaction in partially DA depleted rats is demonstrated morphologically in figure 2 of the appended manuscript. We performed at least two TH immunofluorescent staining experiments in each brain that was analyzed morphologically. Staining for the DA receptors was detected within the thin cytoplasmic rim of medium diameter striatal neurons and occasional large-sized interneurons. Processes also were reactive for the DA receptors, as the striatal neuropil exhibited staining in comparison to the myelinated fiber bundles that penetrate its parenchyma. Only the D2 and D3 receptor subtypes

demonstrated consistent changes at 1 week; D2 protein staining was elevated, while D3 was decreased on the lesioned side in comparison to the control striatum. At the 4 week time point, only the D2 DA receptor staining was reliable in its outcome, and the staining reaction was elevated 20% (N = 14). A representative example of the outcome in partially DA depleted rats is shown in figure 3 of the submitted manuscript (Ariano et al, 2004) and agrees with previous reports using near total striatal DA depletion (c.f., Gerfen et al, 1990 and references therein; Minowa et al, 1994; Hirsch 2000, Hwang et al, 2001).

Task 4: *Evaluate the expression of other neurochemical indices that may be indications of early cellular PD changes in the striatum following unilateral partial DA depletion.* The second messenger cyclic AMP was examined within the striatum, 4 weeks following intrastriatal neurotoxin infusion. Cyclic AMP provides an estimate of basal metabolic rate, since it is intimately involved in the production of ATP. It also transduces D1/D5 DA receptor signaling and may change gene transcription and protein synthesis in target cells. The data suggested that the density of cyclic AMP cells were increased and the intensity of staining likewise was elevated in DA depleted striata compared to intact sides. Typical cyclic AMP elevations in the DA depleted striatum were 25% higher than the values recorded in the intact side ($p < 0.001$, compared using paired, two-tailed student's t-test). Additional components were examined that indicated mitochondrial energy insufficiency and collapse of the potential across the inner membrane occurred in DA depleted striata. A key enzyme in this pathway is caspase-3. Activation of caspase-3 requires proteolytic processing of its inactive zymogen and thus cleaved caspase-3 detection may be an index of pre-apoptotic activity in cells. Our findings showed that caspase-3 cleavage was enhanced in neurons of the DA depleted striatum compared to the intact side. This information is described in the appended manuscript and shown photographically in figure 4 of that paper.

Task 5: *Evaluate the effect of secondary stress on nigrostriatal function using HPLC, 4 weeks following the 6-OHDA infusion.* The subsequent stress experience following the partial unilateral DA lesion caused further losses in the nigrostriatal DA system compared to the untreated rats. We used the predator odorant, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) to provide a species-relevant aversive stimulus (Wallace & Rosen, 2000; Williams 1999). TMT has unique effects on the DA systems of the brain (Morrow et al 2000) and requires no previous shaping of the experimental animals; the vigorous response to the odorant appears innate, even in lab rats. Previous rodent studies have demonstrated clearly that acute emergence of PD behavioral symptoms during stress could be produced following extensive damage of the nigrostriatal DA system (Snyder et al, 1985). Thus, exposure to stressors may lead to significant changes in striatal indices of homeostatic imbalance and further behavioral abnormalities in the preclinical (early) phase of the disease. Rats exposed to TMT exhibited vigorous repulsion to the odor, burrowing in their cage bedding and group-huddling at the opposite end of the cage from the TMT-spotted filter paper. This robust reaction was detected universally throughout the treatment schedule, and describes the rodent fear response (Weninger et al, 1999). HPLC analyses of striatum from the rats exposed to acute (e.g., one TMT treatment followed by sacrifice 24 hours after the exposure), or chronic (5 TMT treatments, delivered once/week, followed by sacrifice 24 hours after the last exposure) schemes were performed. TMT diminished DA and DOPAC levels, but not DA turnover (N = 4 in each group). Statistical significance between acute experimental groups or between the chronic experimental groups for DA levels, DOPAC levels, and the ratio of metabolite to neurotransmitter was compared by ANOVA and Fisher posthoc least squared difference. We also examined limb-use asymmetries in the TMT-treated animals and noted further enhancement in forepaw use ipsilateral to the DA depleted striatum. This provided verification that the DA imbalance was exacerbated by TMT exposure.

03 year 7/01 start through 6/02

Task 1: *Determine the dose of unilateral malonate infusion in SN needed to achieve ~50% loss of striatal DA at 4-weeks after the surgery.* HPLC analysis was used to determine striatal DA and the DOPAC:DA ratio in animals lesioned intranigrally with 1, 2, and 4 μmoles malonate in 1 μl sterile saline, 4 weeks following infusion (N = 8 rats at each dose; Greene and Greena-myre, 1995; Albers et al, 1996).
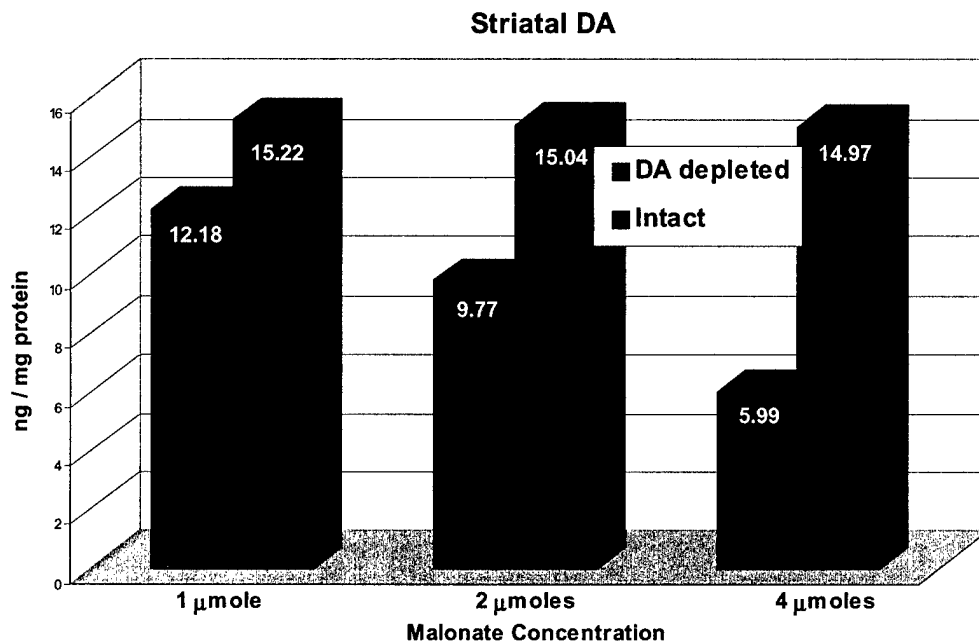
Striatal DA

HPLC Data in Striatum at 4 weeks after 3 μmole SN Malonate
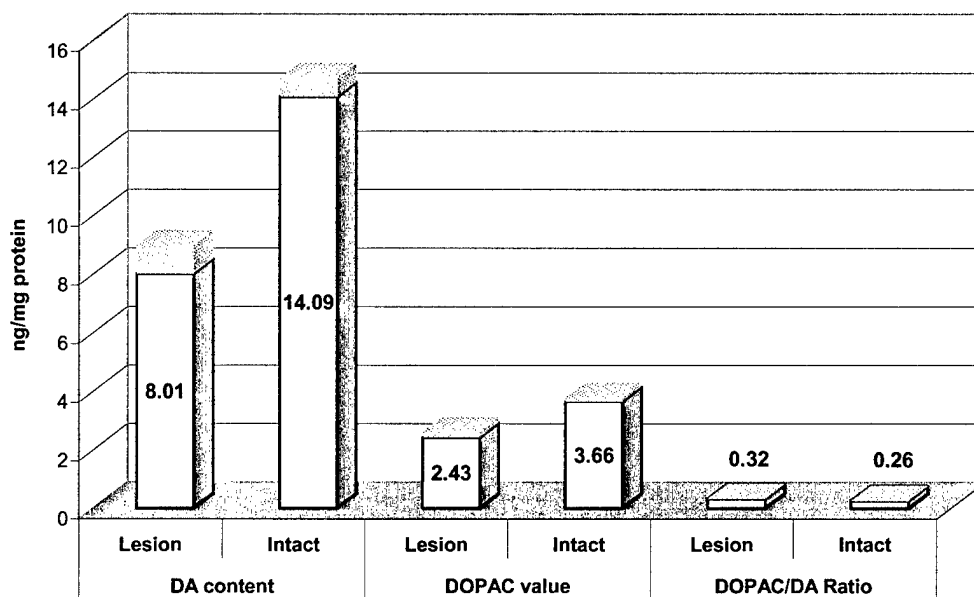
We reduced the concentration of malonate to 3 μmole to better achieve our criterion of 50% reduction in striatal DA. In addition, malonate increased the morbidity of the animals immediately following the surgical intervention; thus we attempted to minimize the concentration of neurotoxin infused in the rats. Animals were evaluated by HPLC (N = 6 each group) or analyzed using immunofluorescence (N = 14). The HPLC analyses were performed in our laboratory.

Task 2: *Behavioral assessment of the DA imbalance was examined in the SN malonate lesioned rats.* Of the 14 animals taken for morphological assessment at 4 weeks following 3 μmole SN malonate infusions, 9 were evaluated for limb-use asymmetries (Tillerson et al, 2001). While there were variations in the percent of usage between individual rats, the forepaw ipsilateral to the intranigral malonate infusion (diagonal bar) was employed more prevalently ($p < .05$) than the forelimb on the intact side (black bar). The behavioral findings were averaged for all nine of the animals over the series of 4 weeks, and compared using two-tailed, paired *t*-test.

Limb-use Asymmetry after SN Malonate Infusion
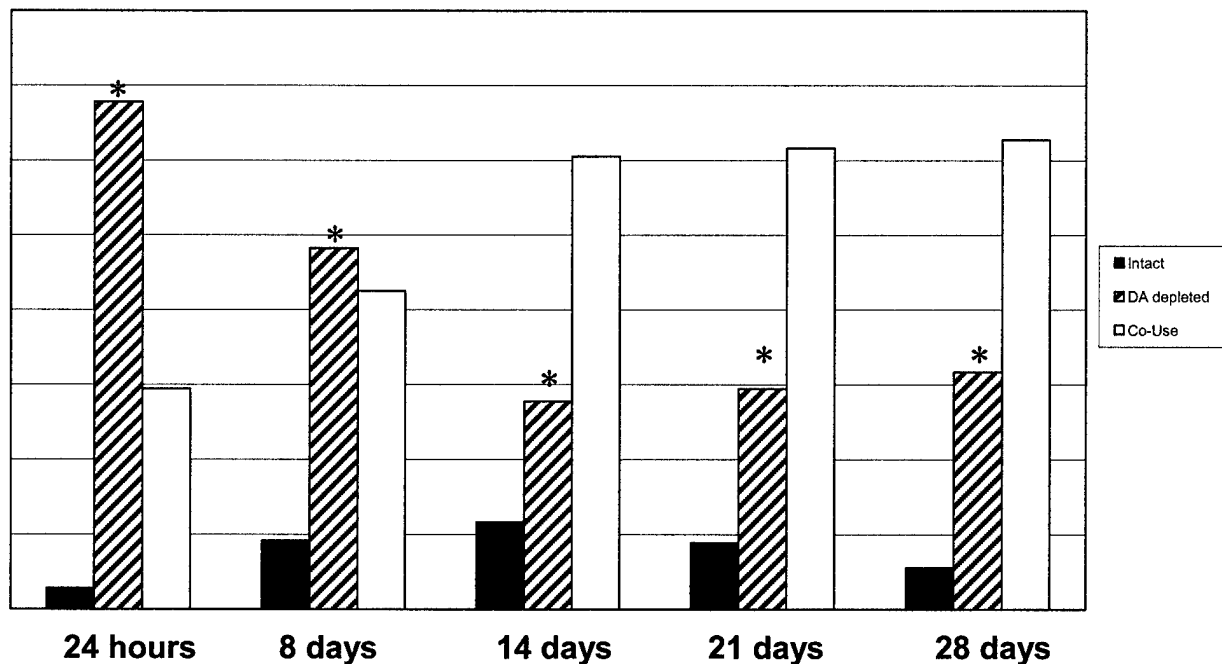
Task 3: *Morphological analysis of the striatum following SN malonate at the 4 week time point was evaluated.* We determined that a consistent elevation occurred in D2 DA receptor and cyclic AMP staining in the malonate rats, analogous to striatal 6-OHDA experiments performed previously. Striatal neurons might be destined for further deterioration due to the alteration in DA homeostasis, and we examined the expression of cleaved caspase-3, a pivotal executioner enzyme in apoptosis leading to subsequent neurodegeneration (Lu et al, 2000; 2001; Mattson, 2000). The response was statistically significant. Partial DA loss produced neuronal elevations in striatal cleaved caspase-3 staining. We extended these studies to evaluate whether the enhanced caspase-3 activity could be associated with specific striatal projection pathways. The data demonstrated significant elevations (+25%) in enkephalinergic striatopallidal cleaved caspase-3 staining within the DA depleted striatum compared to the intact nucleus (N=14).

10

Task 4: *Assessment of changes in the SN malonate treated rats following TMT stressor events.* Rats were divided into TMT-exposed (N = 16) and control (N = 9) groups. Rats exhibited typical fear reactions (Weninger et al, 1999) to the TMT scent, as described previously (Wallace and Rosen, 2001). Animals remained in the isolation room where the TMT was presented until the cages were cleaned, and then they were returned to their home animal room. TMT was presented 1-2 times per week to assure that the experience did not become habituated. Animals were sacrificed after five weeks, and half of the brains were taken for HPLC analysis of the striatal DA and DOPAC levels, the other half were used for morphological evaluation of striatal neurochemical changes. These tissues were stored in the –85°C freezer and were destroyed when the power to the unit was interrupted (see appendix for details). ***These brains had to be replaced in the final period of the grant.***

A pilot study was done to examine whether placement of the neurotoxin affected the outcomes. We had to use 6-OHDA to assure that only the DA nigrostriatal system was lesioned; malonate would poison the electron transport chain in any cell it contacted, regardless of neurotransmitter phenotype and therefore could not be infused into the striatum. 6-OHDA (6 μgm) infusions were made into the striatum (N = 4, 2 μl volume) or the SN (N = 5, 1 μl volume). These rats were videotaped and scored to determine limb-use asymmetries (Tillerson et al, 2001). All animals were exposed subsequently to 5 weeks of TMT, as described above. These samples showed equivalent neurochemical changes in striatal D2 DA receptor, cyclic AMP and cleaved caspase-3 staining, mirroring SN malonate lesioned rats. Changing the neurotoxin placement to the SN alleviated the potential caveat that striatal changes were induced by the infusion of toxin within the nucleus. Complete characterization of the striatal alterations following SN 6-OHDA infusion is presented in the appended manuscript, submitted to *Experimental Neurology*.

Final funded period 7/02 through 1/04 *(We needed to reproduce the brain samples which were lost in the freezer melt down (see appendix Surgical Log), and perform the proposed experiments of the final year of the contract. All of the rats employed in this study are detailed in the appendix, under "Total Census by Paradigm.")*

Task 1: *Replace brains lost in the freezer accident, most of which were to be used for morphological analyses.* Most of the tissues lost had been lesioned using intrastriatal 6-OHDA, and had received the TMT regimen. We replaced some of these brains (N = 12), and also initiated a more extensive use of SN 6-OHDA lesioning paradigm (N = 14). The results mirrored findings obtained using striatal placement of the neurotoxin, and were analogous to data obtained using SN malonate infusions. The outcomes of the partial nigrostriatal loss in the striatum were: 1) Limb-use asymmetry showed preferential use of the forelimb ipsilateral to the lesion which stabilized at 3-weeks after the neurotoxin infusion. 2) TH immunostaining verified the DA imbalance when the animal displayed preferential use of the forelimb ipsilateral to the DA depletion. 3) These differences were not detected in sham animals. 4) D2 DA receptor staining was enhanced in the DA depleted striatum. 5) Cleaved caspase-3 was detected in neurons in the DA depleted striatum. 6) Enzymes upstream (cytochrome C) and downstream (fractin) of cleaved caspase-3 in the intrinsic programmed cell death pathway also were elevated in the DA depleted striatum. The changes were more pronounced in the striatopallidal than the striatonigral pathway (Grissell et al, 2002). This is described in the manuscript appended to this report. **The neurotoxin used, or the placement of the 6-OHDA was irrelevant to the findings; all were equivalent.**

Task 2: *Use western analysis to verify the morphological outcomes on cleaved caspase-3 activity in the DA depleted striatum.* This was completed in 4 different rats and demonstrated robust increases in the protein in the DA depleted striatum. Results were obtained at 4 weeks following SN 6-OHDA infusion. This is presented in figure 4 of the appended manuscript.

Task 3: *Commence the morphological analyses of the TMT experiments to determine whether enhancements occurred in the D2 DA receptor expression and indices of cellular homeostatic imbalance.* These experiments were initiated in previous years, and showed that limb-use asymmetries were enhanced following TMT treatments, DA imbalance was exacerbated by TMT exposure, however we lost most of the morphological analyses for this paradigm when the freezer temperature collapsed. These tissues were re-established, and we are in the process of performing the morphological analyses. Findings in the initial 4 animals examined demonstrate that TH staining is further depressed by TMT exposure, verifying the limb-use asymmetry behaviors. D2 DA receptor and cyclic AMP staining are further elevated in the TMT DA depleted striatum, and cleaved caspase-3 and fractin are enhanced substantially within neurons. We need to complete the analysis of the striatopallidal versus striatonigral expression of the components of the intrinsic programmed cell death pathway. We need to commence verification of our findings using western blots. These experiments are underway in the laboratory.

Personnel issues continued to impact the performance of the research in this contract and are another major reason that the program has not been completed. The research assistant (Agron B. Elezi) hired August 2001 left to attend medical school in May 2002. A college senior student intern (Anne E. Grissell) initiated an independent study project in the spring of 2002, and was hired to replace Elezi in January 2003. Grissell continued to be paid through the end of the award. A second year medical student (Michael J. Maiers) worked for 8 weeks during the summer of 2003 to initiate the western analyses and trained Grissell to perform this assay.

Key Research Accomplishments:

01

- Bilateral striatal lesions produced severe dietary consequences requiring caloric supplementation using specialized liquid diets and increased the morbidity of the rats
- Intrastriatal infusion of 6-OHDA (6.25 µgm/4 µl total) in four different striatal sites (2 per side) produced ~50% loss of striatal DA upon HPLC analysis but increased morbidity
- 24-hour behavioral assessments could not be performed due to the status of the rats, immediately following the neurotoxin infusion
- A single behavioral paradigm (Montoya staircase apparatus) was sensitive enough to detect skilled forepaw use, balance and postural deficits following partial bilateral loss of striatal DA

02

- Unilateral infusion of 6 µgm (2 µl into 1 striatal site) of 6-OHDA produced a statistically significant loss of DA in the striatum that was reproducible and the health of the animals was

robust through the experimental time line. HPLC values showed a range of 30-85% loss with the average ~50% -- the criterion we chose to examine early (preclinical) changes in PD rats.

- Morphological assessment of the striatum 1 week following 6-OHDA treatments demonstrated nearly equivalent staining patterns and intensity for the D1, D4, and D5 DA receptor proteins. D3 DA receptor protein was decreased on the side of the neurotoxin infusion. The D2 DA receptor was elevated on the neurotoxin-treated side, as was cyclic AMP. DA terminal losses were assessed using TH immunofluorescence and showed a substantial decrement in staining within the neurotoxin-treated striatum compared to the intact side.

- Unilateral infusion of this same amount of neurotoxin (6 μgm in 2 μl into 1 striatal site) produced ~50% loss of DA 4 weeks after the neurotoxin treatment as determined using HPLC.

- Morphological evaluation of the striatum 4 weeks after the 6-OHDA infusion demonstrated that the D2 DA receptor and cyclic AMP staining were still elevated at this time point within the neurotoxin–treated striatum compared to the intact side. No other changes were detected using immunofluorescence for the other DA receptor subtypes.

- Treatments with the species-relevant predatory odor TMT showed remarkable behavioral avoidance by the exposed rats. Two experimental designs were used, an acute 24-hour survival, and chronic 5 week repetitive weekly exposure sequence. The HPLC analysis showed that the odorant decreased DA and DOPAC levels.

- The 50% DA depletion produces profound effects on the pre-apoptotic enzyme caspase-3 in the 4 week intrastriatal 6-OHDA animals. Statistically significant 40% increase in the expression of cleaved caspase-3 in the DA-deafferented striatum occurred, and this elevation predominated in neurons. There was no cell loss, and thus apoptosis did not occur. Slight reduction in the diameter of the striatum occurred but was not significant. This finding indicated some atrophy might have occurred in striatal neurons. Alternatively, this may be a confound of the neurotoxin infusion into this site.

03

- The species relevant odorant stressor TMT used in a mild regimen, one hour-long exposures twice per week, for 4-5 weeks, 4 weeks following intrastriatal 6-OHDA DA depletion produced an accelerated loss in striatal DA levels and its turnover (DOPAC:DA ratio). These results were statistically significant in comparison to non-TMT treated 6-OHDA-lesioned rats.

- Intranigral infusion of malonate caused a substantial acute behavioral response in all rats, regardless of the dose infused. Use of 3 μmole intranigral malonate (in 1 μl) provided the criteria of ~50% loss of striatal DA, 4 weeks post-infusion as assessed by HPLC.

- Rats lesioned with this dose of malonate demonstrated significant limb-use asymmetry in that the forepaw ipsilateral to the DA depletion was preferred for exploring a vertical surface.

- Morphological assessment of intranigral malonate-lesioned rats was analogous to findings detected previously following intrastriatal 6-OHDA infusions. We detected loss in TH staining; elevations in D2 DA receptor staining, elevations in the number of cyclic AMP stained cells and their intensity, and enhanced neuronal cleaved caspase-3 expression. All staining was statistically significant in the DA depleted striatum compared to the intact side.

- Extending the studies on cleaved caspase-3 showed that some of the elevated staining intensity was associated specifically with the indirect, enkephalinergic striatopallidal pathway.

- Cleaved caspase-3 protein levels were enhanced robustly in the DA depleted striatum, determined using western analysis.

04

- Intranigral malonate infused rats were exposed to TMT. HPLC, morphology, and behavioral analyses mirrored the outcomes established using intrastriatal 6-OHDA infusions. TH staining was reduced; D2 DA receptors, cyclic AMP, cleaved caspase-3, cytochrome C, and fractin were all elevated within the DA depleted striatum compared to the intact side.
- We correlated placement of the 6-OHDA neurotoxin with the behavioral and morphological outcomes, and determined that the results were analogous regardless of where the nigrostriatal pathway was interrupted. It is less problematic to infuse the neurotoxin within the substantia nigra, as this reduces the potential damage to the striatum by infusion of liquid and more evenly affects the striatal neurons.
- TMT treatment exacerbates the changes of cellular homeostatic indicators in the striatum, impacted by the partial DA depletion. This has been monitored using HPLC, limb-use asymmetry measurement, morphological evaluation, and western analysis.

Reportable Findings/Presentations/Funding

- Grissell AE, Buchanan TM, **Ariano MA** (2002) Striatopallidal changes in a preclinical rat model of Parkinson's disease. *Ann NY Acad Sci.* **991**:278-280.
- **Ariano MA,** Littlejohn FC, Buchanan TM, Collier KS (2002) Neurochemical and behavioral changes in a preclinical rat model of Parkinson's disease. *Soc Neurosci, Program No.* **226.3.**
- Littlejohn FC, **Ariano MA** (2002) Caspase-3 activity is increased in striatal neurons after subtotal 6-OHDA nigrostriatal lesions. *Chicago Chapter of Soc Neuroscience, poster session.*
- Buchanan TM, **Ariano MA** (2002) Behavioral and neurochemical changes in rats following partial nigrostriatal DA depletion: cleaved caspase-3 and 8-oxoguanine. *Chicago Chapter of Soc Neuroscience poster session.*
- Grissell AE, Maiers MJ, **Ariano** MA (2003) Striatopallidal apoptosis in early Parkinson's disease? *Chicago Interdepartmental Program in Neuroscience Retreat poster session.*
- **Ariano MA,** Grissell AE, Littlejohn FC, Buchanan TM, Maiers MJ, Elsworth JD, Collier TJ, Collier KS (2004) Striatal changes in experimental preclinical Parkinson's disease: Behavioral and neurochemical characterization after partial dopamine depletion. *Exp Neurol,* in review.
- **Ariano MA,** Grissell (2004) Early striatopallidal changes after partial dopamine depletion affect the intrinsic programmed cell death pathway. *Intl Basal Ganglia Society poster session.*
- A preclinical Parkinson's disease model: unexpected striatal changes. Seminar presentation in the Department of Cellular and Molecular Pharmacology, Chicago Medical School. October 2002.
- A competitive renewal, "Striatal changes in experimental preclinical PD" was applied for from the DOD, September 2002 (Ariano as PI). This was not funded.
- Funding has been applied for at the NIH: NS046391, "Striatal changes in experimental preclinical PD." A revision is contemplated for the Oct 04 session (Ariano as PI).
- Pilot project feasibility study for institutional funds has been applied for entitled, "Characterization of anxiety and stress in nucleus accumbens using a rodent model of early Parkinson's disease." (Atre-Vaidya as PI and Ariano as co-PI)

Conclusions and Significance of the Findings

Our data suggest that substantial behavioral and neurochemical changes occur following partial interruption of the DA nigrostriatal pathway to produce the preclinical (early stage) PD rat model. These changes are detectable behaviorally (limb-use asymmetry) and morphologically (cyclic AMP signaling changes, and apoptosis indices), and have been confirmed using HPLC and western analyses. The observation of heightened expression in the apoptosis pathway is unique. I was very skeptical of the data when we first detected these findings, but the consistent changes of initial and late enzymes in the pathway have convinced me that these outcomes are valid. I think the appearance demonstrates imbalances in striatal cellular homeostasis – it is NOT an indication of apoptosis. We assessed changes in enzymatic activity of upstream and downstream proteins in identified neurons, resulting from activity of the apoptosome. The cytoplasmic appearance of cleaved caspase-3 confirms that the intrinsic pathway has been activated; that the apoptosome formed, and was enzymatically active because the product (cleaved caspase-3) was elevated in the DA depleted striatal neurons.

At the extreme, cleaved caspase-3 may initiate programmed cell death in severely damaged neurons (Blum et al, 2001; He et al, 2000; Lu et al, 2000; 2001; Mattson, 2000; Nagatsu, 2002; Qin et al, 2000; Tatton, 2000). We do not detect neuronal losses in the DA-depleted striatum. Consequently appearance of cleaved caspase-3 may be a transient event and striatal neurons may recover partially if given sufficient cellular support and/or time to heal without further secondary insults. This warrants further study. A recent report showed that neuroprotection can be conferred by cleaved caspase-3 after partial ischemic insults (McLaughlin et al, 2003). An analogous situation may underlie the increased activities we detected in striatopallidal neurons. Cleaved caspase-3 may actually be signs of cellular stress, precipitated by the collapse of the mitochondrial membrane potential. Additional data report that increased cyclic AMP levels can delay caspase-3 induction of apoptosis (Moran et al, 1999). We see robust elevations of cyclic AMP within striatal neurons. Taken together these outcomes make us think that the appearance of apoptosis enzymes does not signal impending neurodegeneration in specific striatal outputs.

Other data shows that these neurochemical changes are not expressed in the striatonigral pathway. This argues for the functional opposition of striatal projection systems, as contended in earlier works (Albin et al, 1989; Gerfen et al, 1990). Dysfunctions which may be caused may include hyperexcitability (Calabresi et al, 1993), impaired energy production (Blandini et al, 1996; Greenamyre et al, 1999) and breakdown of the actin cytoskeleton. Enhanced caspase-3 protein staining also exhibited heightened enzymatic activity since its breakdown product fractin was detected. Fractin was elevated significantly in the DA depleted striatum compared to the intact side. Remodeling the cytoarchitecture of striatal neurons would occur as actin is fragmented by caspase-3 cleavage, and reports have demonstrated that DA loss will indeed reshape the dendritic structure and spine density of striatal projection neurons (Ingham et al, 1988; Arbuthnott, 2000; Dervan et al, 2002), contributing to differences in neuronal responsiveness.

Thus, partial nigrostriatal disruption causes substantial changes in the terminal field area, long before obvious motor symptoms of PD would be detected in the clinic. Evidence, both published and anecdotal, describes augmentation of PD symptoms in patients and animal models of the disease under stressful conditions (Snyder et al, 1985; Frazier, 2000; Factor and Weiner, 2002). Introduction of a stress event to our preclinical PD rat model should exaggerate these changes. We detected enhancement in limb-use asymmetries that indicated further exacerbation of striatal DA terminal loss. Acute exposure of rats to TMT stimulates the stress response (Morrow et al, 2000). We have extended those investigations and the outcomes clearly demonstrate further deterioration in striatal DA. The indicators of mitochondrial membrane potential collapse

also were elevated further by the TMT treatment regimen. In summary our data provide a different avenue of potential therapeutic intervention; perhaps the use of inhibitors of caspase cleavage would retard the insidious deterioration of the basal ganglia, and at least prolong the time before the necessity of using L-DOPA therapy in patients.

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Appendix Materials

Finch University of Health Sciences / The Chicago Medical School

Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

28 November 2000

Ms. Judy Pawlus
Office of the Deputy Chief of Staff
   Information Management Branch
Department of the Army
US Army Medical Research and Materiel Command
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Ms. Paulus

I am writing in reference to my award, # DAMD17-99-1-9542. It has become apparent to me that the original research plan in this award is hindering our progress and not allowing us to investigate fully the questions that we have proposed to address. After spending considerable time at the recent Society for Neuroscience meeting in New Orleans speaking with colleagues and experts in the field regarding specific concerns, I would like to propose a revision to the research plan. I believe the revision outlined below will assure results that are more consistent with current behavioral studies. The rationale for this change is noted below with justifications.

1. Unilateral lesions of the dopaminergic nigrostriatal pathway should be employed rather than a bilateral design. While Parkinson's disease in humans does develop initially as a unilateral disturbance and then progresses to bilateral involvement, the unilateral rodent lesion model has given useful information for the past 40 years and is the gold standard for animal studies of the movement disorder. The ability to produce a dopaminergic chemical imbalance in the nigrostriatal pathways by unilateral lesioning enables testing of specific dopaminergic behaviors in rodents that can be quantified readily.

2. Bilateral lesions have mandated special dietary and husbandry requirements that have delayed progress of our research. It also is evident that these special needs impede the use of two of the originally proposed time points (e.g., 24 hours and 3 days). Unilateral lesions are less challenging to the animals' post-surgical recovery. No special dietary considerations need to be implemented, either before the lesioning to introduce the diet to the animals, nor afterwards to maintain their body weights.

3. A unilateral lesion will provide an internal control in that the contralateral side can be used to assess changes in various morphological and chemical parameters, instead of using a naïve/control group of rats. This will decrease the number of animals necessary to perform the study.

4. While we have established the amount of striatal 6-hydroxydopamine that can be used to provide a bilateral 50% loss of striatal dopamine one week following the surgery, our progress has been slow due to a number of issues. Paramount among these deterrents is the inconsistency of the bilateral lesions within a single animal, regardless of the experience of the surgeon producing the neurotoxin depletion of the transmitter.

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Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

It is absolutely imperative that we perfect the lesioning task before initiation of behavioral testing of the animals. If we cannot ensure reliable and consistent 50 % losses of striatal dopamine using the two different neurotoxin infusion paradigms (6-hydroxydopamine in the striatum and malonate in the substantia nigra), behavioral testing will not have a meaningful outcome. Thus the lesion paradigm is the first step of this project.

We are in the process of establishing collaboration with an expert in the field of dopamine behavioral assessment, Dr. Timothy Schallert (University of Michigan). He will teach us to quantify nigrostriatal dopaminergic losses using a very simple, straightforward behavior that assesses the number of paw touches by the animal in a vertical Plexiglas cylinder. Rats do not use the paw on the dopamine lesioned side compared to the intact dopaminergic nigrostriatal pathway, and Dr. Schallert has correlated this relationship with the chemical loss of the neurotransmitter levels in the striatum. This experimental design precludes the need to shape the animals' behavior on the task prior to the surgery. Moreover the natural tendency for the rodent to explore the cylinder will allow us to assess the rats' depletion without food deprivation to motivate the behavioral sequence. Thus we will be able to determine the extent of the dopaminergic depletion prior to sacrifice of the animal and not as a retrospective analysis. All of these amendments to our present experimental design will speed the progress of the research, improve the consistency of our data, and importantly, maintain a robust health in our lesioned animals.

We would like to initiate these changes quickly so that we will have an interesting and coherent report to share with our colleagues at the upcoming March 2001 meeting in Potomac, MD. Thank you for your cooperation and assistance in this matter.

Sincerely,

Marjorie A. Ariano, Ph.D
Professor of Neuroscience
Principal Investigator for the Project

Kathy Steece-Collier, Ph.D
Assistant Professor of Neuroscience
Co- Principal Investigator for the Project

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Marjorie Ariano

From: "Friedl, Karl E LTC USAMRMC" <Karl.Friedl@DET.AMEDD.ARMY.MIL>
To: "'Marjorie Ariano'" <Marjorie.Ariano@finchcms.edu>
Cc: "Yourick, Debra L Dr. WRAIR-Wash DC" <Debra.Yourick@NA.AMEDD.ARMY.MIL>; "Pawlus, Judy K Ms USAMRMC" <Judy.Pawlus@DET.AMEDD.ARMY.MIL>; "Miles, Cheryl R Ms USAMRAA" <Cheryl.Miles@DET.AMEDD.ARMY.MIL>; "Grate, Stephen J Dr SHERIKON" <Stephen.Grate@DET.AMEDD.ARMY.MIL>; "Burdette, Buffy J Ms SHERIKON" <Buffy.Burdette@DET.AMEDD.ARMY.MIL>
Sent: Thursday, April 05, 2001 4:43 PM
Subject: RE: "SOW" DAMD17-99-1-9542

If I understand your request correctly (28 Nov 00), you asked to switch to unilateral lesioning and noted a need to perfect your lesioning technique. You also asked to add Dr. Schallert from the University of Michigan as a consultant to help with your behavioral measurement techniques. There is no change in budget and no change in schedule. If this is the case, this email can certify that we will accept your experimental modifications without further delay; these are sensible adjustments.

Ms. Cheryl Miles in the contracting office may formalize this with a contract modification although I don't believe that will be necessary; Ms. Judy Pawlus is the correct point of contact for your periodic progress reports; and Dr. Debra Yourick at Walter Reed Army Institute of Research is your technical point of contact as the Contracting Officer's Representative.

On your previous annual report, Dr. Yourick noted that you had some experimental setbacks but felt that everything was properly executed and was scientifically acceptable. An outside review that we also conduct on each annual report was a bit harsher in their assessment of progress (you should have received a copy of that review) but you have assured me that you are making good progress and will be on schedule to meet all of your research objectives. Please feel free to discuss technical issues and concerns with Dr. Yourick (copied on this email) as you progress. We are interested in helping you to succeed and it sounds like you are on the right track.

Thank you again for taking time from your schedule to participate in the workshop. We have received enough favorable comments that we will definitely consider a reprise if the program continues to grow.

Karl Friedl

-----Original Message-----
From: Marjorie Ariano [mailto:Marjorie.Ariano@finchcms.edu]
Sent: Thursday, April 05, 2001 2:47 PM
To: karl.friedl@det.amedd.army.mil
Cc: stephen.grate@det.amedd.army.mil
Subject: "SOW"

LTC Friedl,

I still have not received anything in writing regarding the approval of my amended SOW. I presume it is "in the mail?"

Marjorie A. Ariano, Ph.D
Professor
Neuroscience
The Chicago Medical School
3333 Green Bay Road
North Chicago, IL 60064-3095 USA

REPLY TO
ATTENTION OF:

April 11, 2001

R & D Branch

SUBJECT: DAMD17-99-1-9542
           Modification No. P00001

Mr. John W. Gantz
Executive Vice President for Finance
The Chicago Medical School
3333 Green Bay Road
North Chicago, Illinois   60064-3095

Dear Mr. Gantz:

Enclosed are three (3) copies of the above subject grant modification. Two (2) copies should be signed by an official authorized to bind your organization and returned to this office. The remaining copy should be retained for your file. However, if questions arise, which preclude your timely execution of the document in its present form, please contact Mrs. Sherry Labella, Contract Specialist at 301-619-2806 or e-mail at:
sherry.labella@det.amedd.army.mil.

Sincerely,

Melanie S. Harman
Procurement Technician

Enclosures

ASSISTANCE AGREEMENT

AWARD TYPE: ☑ GRANT (31 USC 6304)	☐ OPERATIVE AGREEMENT (31 USC 6305)	☐ ER TRANSACTION (10 USC 2371)

AWARD NO: DAMD17-99-1-9542 Modification No. P00001	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT $414,198.00	Page 1 of 1 POC: Cheryl Miles Tel: 301-619-7148 Fax: 301-619-3166

PROJECT TITLE: Degenerative Risks for Parkinson's
Disease after Toxin Exposure and Stress

CFDA 12.420

PERFORMANCE PERIOD: 1 Jul 1999 - 1 Aug 2002 (Research Ends 1 Jul 2002)	PRINCIPAL INVESTIGATOR: Dr. Marjorie Ariano

AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-A 820 Chandler St. Fort Detrick Maryland 21702-5014	PAYMENTS WILL BE MADE BY: EFT:T DFAS - SA/FPA 888-478-5636 - Tel ATTN: Army Vendor Pay 210-527-8189 - Fax 500 McCullough Ave. San Antonio, TX 78215-2100

DUNS No:	TIN No:	(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)

AWARDED TO: Finch University of the Health Sciences The Chicago Medical School 3333 Green Bay Road North Chicago, IL 60064-3095	REMIT PAYMENT TO: Same as Awarded To address

ACCOUNTING AND APPROPRIATION DATA: NA

SCOPE OF WORK:

1. Work under this award shall be accomplished in accordance with the revised scope of work, dated 28 November 2000, which is incorporated herein by reference.

2. All other terms and conditions are unchanged.

RECIPIENT	GRANTS OFFICER
ACCEPTED BY:	UNITED STATES OF AMERICA
_____ SIGNATURE	_____ SIGNATURE

NAME AND TITLE	DATE	NAME AND TITLE Cheryl R. Miles GRANTS OFFICER	DATE

Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

28 March 2002

Ms. Wendy Cockerham
Contract Specialist
US Army Medical Research
MCMR-AAA, NETRP
820 Chandler Street
Fort Detrick, MD 21702-5014

DAMD 17-99-1-9542

Dear Ms. Cockerham,

I am requesting a no-cost extension for my proposal entitled, "Degenerative Risks for Parkinson's Disease after Toxin Exposure and Stress." I would ask that the Army extend the duration of the project for <u>18 months</u>, and my rationale for this extension is provided below. In addition, I propose a change in the SOW to reflect the alteration in duration and scope of the project.

- **Personnel issues.** In the course of the past two and one half years we have had a very difficult time identifying appropriate laboratory technical help. This has substantially slowed the progress of the present award, as the day-to-day running of the lab has been inconsistent without this type of individual on-board. A new research assistant was hired in August 2001. As a consequence, large numbers of animals are infused with neurotoxin at a single time, using the technical expertise of two colleagues at Rush-St. Luke's Presbyterian Medical Center in Chicago (Dr. T.J. Collier and Mr. B. Daley). This has enabled us to evaluate the neurotoxin effects on nigrostriatal functioning much more thoroughly, although there is still a "bottle-neck" in completing our morphological analyses. It has become apparent that I must perform this analysis, as the research assistant does not have the background to make judgments on the morphological outcomes of the experiments. I am not always available to facilitate this aspect of the research due to other commitments and responsibilities. This will necessitate increasing the duration of the research program to complete these morphological studies. Because we experienced gaps in our staff employment, we have sufficient funds to cover the salary of a ¾ time lab assistant through the next year.

- **Departure of the co-PI.** As of 15 April 2002, the co-PI on the grant, Dr. Kathy Collier will be leaving the Chicago Medical School. Dr. Collier will not receive any further salary compensation from this point forward, providing additional funds to support continued technical assistance for the project. Dr. Collier will be relocating to Rush-St. Luke's Presbyterian Medical Center in Chicago, and thus will still be able to provide input to the scientific scope of the project.

- **Freezer debacle.** During the weekend of December 8-9, 2001, the electrical circuit breaker on the generator-backed up power line that serves my –85°C freezer was tripped, terminating electricity to the unit. The loss of power was not reported to me during the weekend, and thus was not discovered until I arrived at work on Monday morning. The internal temperature of the freezer was at 0°C by that point, and the inside was dripping with moisture. There were 26 freezer towers with 7-9 boxes containing custom produced antisera, peptides, and animal brain tissues accumulated over the past 20 years. Four towers contained brain tissue prepared for the DoD neurotoxin program 02 year studies, which were lesioned and had been exposed to a predator odorant as a secondary stressor event (as well as an experimental transgenic Huntington's disease brain bank). The brain tissues did ***not*** survive the increase in temperature, and cannot be used for the planned morphological analyses. We are thus re-preparing this tissue for the current proposal in addition to the planned 03 year complement of experiments, and this will take additional time to complete. We have retained the thawed and refrozen samples, and are in the process of assessing whether or not they are viable for biochemical analyses, such as western determination of protein levels. The University has provided monies to directly replace the cost of animals and their per diem, but this hardly covers the loss of nearly 1 year of research, and the stunning setback in time and morale.

These three reasons therefore, have motivated this request for an 18-month extension to complete the proposed investigations of Parkinson's disease and secondary stressor events.

This will necessitate a change in the SOW since the time frame has been altered considerably. I would propose the following measures, detailed below.

We had approximately 40 brains from the experimental paradigms used in the 02 year of our research program that no longer have sufficient integrity to analyze morphologically, I propose that we attempt to perform western assays for dopamine receptor levels on that tissue. We have begun to establish the appropriate protocols for this purpose using our D1 dopamine receptor specific antisera, and the results look promising. I anticipate we also will be able to establish protein levels for the D2 receptor in these tissues, since both D1 and D2 receptor proteins exist in sufficient quantity to detect in whole striatal homogenates. However, I do not think it is feasible to expect consistent detection for the D3, D4, or D5 dopamine receptor proteins due to low protein levels in a full striatal homogenate. We will at least attempt this method on intact rats before abandoning the approach. If we cannot detect D3, D4, and D5 dopamine receptors using westerns, we will re-prepare the 02 year brains for sufficient morphological evaluations to provide statistical confidence using luminosity measurements and qualitative trends in the results.

Our survey to evaluate other potential cellular mechanisms underlying postsynaptic changes in the nigrostriatal pathway following neurotoxin exposure has elucidated some intriguing preliminary data. We have demonstrated that infusion of the neurotoxin 6-OHDA into the striatum, to yield a subtotal dopamine depletion lesion (6 µgm in 2 µl into the striatum, as described in the 01 and 02 year progress reports), produced an elevation in the active form (e.g., cleaved) pre-

apoptotic enzyme, caspase-3, at four weeks after the neurotoxin lesion (N = 4 rats). This was most intriguing, as it suggested to us that a mild insult to the dopamine afferents would predispose the striatal neurons, which are the primary targets of drug therapy in Parkinson's disease, to cellular malfunction and eventual degeneration. A potential confound of our paradigm was that infusion of the neurotoxin could substantially damage the parenchyma of the striatum, and that in turn could predispose the striatal cells to apoptosis. We have extended our investigation of this phenomenon, and determined that infusion of 6-OHDA into the substantia nigra (6 μgm/2 μl) also produces the same elevation in cleaved caspase-3 in the postsynaptic striatum at the four week time point (N – 4 animals). We further determined that the elevation of the cleaved caspase-3 occurred in neurons, using the simultaneous detection of NeuN, a neuron-specific marker (N = 4 animals). Further, it appears that the elevation of caspase may be associated specifically with the enkephalin producing, indirect striatopallidal system, which demonstrates enhanced functioning following losses of nigrostriatal dopamine (N = 2 animals). We propose to pursue these investigations in the extended grant period and evaluate the time course of this change, and if other markers of apoptosis or oxidative stress occur in particular striatal neuron populations. We propose to examine cytochrome oxidase complex 1 (COX1) and 8-oxoguanine (a marker of reactive oxygen species damage to DNA) in these studies following unilateral 6-OHDA nigral infusions. These studies will be assessed using double immunofluorescence staining and we also will attempt to analyze the changes quantitatively using western analyses.

We need to reproduce the secondary stressor experiments following neurotoxin infusions using both 6-OHDA and malonate. We propose to establish a more frequent exposure regimen as our preliminary HPLC findings suggested that one hour per week exposure to the predator odor, TMT was insufficient to produce changes in striatal or nigral dopamine levels or turnover, even though the animals exhibited robust behavioral responses to its presentation. We will evaluate these experiments using HPLC, dopamine receptor and cyclic AMP immunofluorescence in the striatum, and specific expression of cleaved caspase-3 staining in the enkephalin striatopallidal pathway using double immunofluorescence techniques.

We have begun to video tape the behavior of our neurotoxin lesioned animals, using the forepaw placement rating described by Dr. T. Schallert (Tillerson et al, *J Neurosci* 21:4427, 2001). We are hopeful this will demonstrate subtle behavioral changes that occur with the subtotal nigrostriatal dopamine lesions to enable us to relate the biochemical and morphological differences with a phenotype.

There are potential problems that might preclude the successful completion and performance of these proposed experimental approaches. The most obvious confound is the low level of protein expression for the D3, D4, and D5 dopamine receptors, which may preclude their detection using western analysis. We will need to examine this in the upcoming months. We are still evaluating the appropriate TMT exposure regimen that will initiate a biochemical and morphological change in the nigrostriatal system, without habituating the animals to the stressor event. I am sure there are other considerations we have not encountered that could impact on the success-

Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

ful performance of these renewed aims. We will re-evaluate our paradigms as we encounter them, and keep the DoD apprised of our progress.

Thank you for your time and attention. If there are other items of information that you need, please contact me by telephone at (847) 578-3412, fax (847) 578-8515, or by email using arianom@finchcms.edu.

Sincerely,
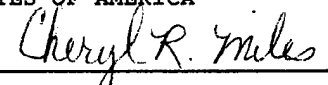
Marjorie A. Ariano, Ph.D
Professor, and PI of the project

Velayudhan Nair, Ph.D, D.Sc
VP of Research and
Dean School of Graduate and
Postdoctoral Studies

ASSISTANCE AGREEMENT

2303

AWARD TYPE: ☑ GRANT (31 USC 6304)	☐ OPERATIVE AGREEMENT (31 USC 6305)	☐ ER TRANSACTION (10 USC 2371)

AWARD NO: DAMD17-99-1-9542 Modification P00002	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT $414,198.00	Page 1 of 1 Wendy A. Cockerham 301-619-2034/phone 301-619-4084/fax

PROJECT TITLE: "Degenerative Risks for Parkinson's Disease after Toxin Exposure and Stress"

CFDA 12.420

PERFORMANCE PERIOD: 01 Jul 1999 - 01 Feb 2004 (research ends 01 Jan 2004)	PRINCIPAL INVESTIGATOR: Dr. Marjorie Ariano

AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-A 820 Chandler St. Fort Detrick Maryland 21702-5014	PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA/FPA 500 McCullough Avenue San Antonio, TX 78215-2100
DUNS No: 01-721-1439 TIN No:	*(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)*

AWARDED TO:
Finch University of the Health Sciences
  The Chicago Medical School
3333 Green Bay Road
North Chicago, IL 60064-3095
	REMIT PAYMENT TO:
	Finch University of the Health Sciences
	  The Chicago Medical School
	3333 Green Bay Road
	North Chicago, IL 60064-3095

ACCOUNTING AND APPROPRIATION DATA:
not applicable

SCOPE OF WORK:

A. The purpose of this modification is to extend the period of performance to read as shown above. This is being done at no additional cost to the Government and in accordance with the recipient's request dated 28 March 2002. The period of performance is hereby changed

FROM: 01 July 1999 - 01 August 2002 (research ends 01 July 2002)
  TO: 01 July 1999 - 01 February 2004 (research ends 01 January 2004)

B. All other terms and conditions remain unchanged.

RECIPIENT	GRANTS OFFICER
ACCEPTED BY: signature not required in accordance with recipient's letter dated 3/28/02 _____ SIGNATURE	UNITED STATES OF AMERICA *Cheryl R. Miles* _____ SIGNATURE
NAME AND TITLE DATE	NAME AND TITLE DATE Cheryl R. Miles 5/20/02 GRANTS OFFICER

REPLY TO
ATTENTION OF:

.8 1 MAY 2002

R & D Contracts Branch (WAC/cfs)

Mr. John W. Gantz
Executive Vice President
  for Finance
The Chicago Medical School
North Chicago, IL 60064-3095

Dear Mr. Gantz:

    Enclosed is a copy of Modification No. P00002 to Grant
No. DAMD17-99-1-9542 fully executed, for your information and
file.

    If you have any questions regarding the administration
of the referenced grant, please contact the undersigned at
301-619-2034 (by facsimile at 301-619-4084 or by e-mail at
wendy.cockerham@amedd.army.mil).

    An extra copy of the modification is provided for the
principal investigator.

                              Sincerely,

                              Christine Suan

                         for Wendy A. Cockerham
                              Contract Specialist

Enclosures

Total Census By Paradigm

Experiment	Analysis	Count
1 wk bilat Str 6-OHDA	HPLC	16
1 wk bilat Str sham	HPLC	3
1 wk uni Str 6-OHDA	HPLC	9
	IHF	20
1 wk uni SN 6-OHDA	IHF	4
1 wk uni SN malonate	IHF	4
4 wk uni Str 6-OHDA	HPLC	16
	IHF	30
4 wk uni Str sham	IHF	2
4 wk uni SN malonate (1 μmole)	HPLC	3
4 wk uni SN malonate (2 μmole)	HPLC	6
4 wk uni SN malonate (4 μmole)	HPLC	2
4 wk uni SN mal @ 1,2,4 μmole	IHF	1 @ each dose (3)
4 wk uni SN malonate (3 μmole)	HPLC	27
	IHF	19
4 wk uni SN 6-OHDA	HPLC	
	IHF	14
4 wk uni Str 6-OHDA/1 TMT	HPLC	6
no TMT	HPLC	4
4 wk uni Str 6-OHDA/5 wk TMT	HPLC	9
no TMT	HPLC	4
4 wk uni Str 6-OHDA/5 wk TMT	IHF & blot	23
no TMT	IHF & blot	8
4 wk uni SN 6-OHDA/5 wk TMT	IHF& blot	22
no TMT	IHF & blot	25
sham lesion/5 wk TMT	IHF& blot	4
sham lesion/no TMT	IHF& blot	3
4 wk uni SN 6-OHDA/10 wk TMT	IHF& blot	8
no TMT	IHF& blot	8

Total rats used **302**

Surgical Log

	A	B	C	D	E	F	G	H
1	Date	Surgeon & rat	surgery	sac date	toxin dose	experiment	status	Notes
2	9/11/2000	Tim Collier #1	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
3		Tim Collier #2	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
4		Kathy Collier #3	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
5		Tim Collier #4	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
6		Kathy Collier #5	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
7		Kathy Collier #6	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
8		Tim Collier #7	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
9		Kathy Collier #8	bi-lat CN 6-OHDA	9/18/2000	2.5 mg/ml; 2 µl/CPu	HPLC	done	
10	9/21/2000	Laura Stanisz #9	bilateral-sham	9/29/2000		HPLC	done	
11		Laura Stanisz #10	bilateral-sham	9/29/2000		HPLC	done	
12		Laura Stanisz #11	bilateral-sham	9/29/2000		HPLC	done	
13								
14	10/11/2000	Tim Collier #1	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
15		Kathy Collier #2	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
16		Tim Collier #3	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
17		Kathy Collier #4	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
18		Kathy Collier #5	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
19		Tim Collier #6	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
20		Tim Collier #7	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
21		Tim Collier #8	bi-lat CN 6-OHDA	10/18/2000	3 mg/ml; 2 µl/CPu	HPLC	done	
22								
23	12/5/2000	Kathy Collier #1	unilat CN 6-OHDA	12/12/2000	3 mg/ml; 2 µl/CPu	IHC	20.7	
24		Margie Ariano #2	unilat CN 6-OHDA	12/12/2000	3 mg/ml; 2 µl/CPu	IHC	died	
25		Kathy Collier #3	unilat CN 6-OHDA	12/12/2000	3 mg/ml; 2 µl/CPu	IHC	20.7	
26		Margie Ariano #4	unilat CN 6-OHDA	12/12/2000	3 mg/ml; 2 µl/CPu	IHC	20.7	
27								
28	12/13/2000	Kathy Collier	unilat CN 6-OHDA	12/20/2000	3 mg/ml; 2 µl/CPu	IHC	20.3	
29		Kathy Collier	unilat CN 6-OHDA	12/20/2000	3 mg/ml; 2 µl/CPu	IHC	20.3	
30								
31	12/20/2000	Margie Ariano #1	unilat CN 6-OHDA	12/28/2000	3 mg/ml; 2 µl/CPu	IHC	21.8	
32		Margie Ariano #2	unilat CN 6-OHDA	12/28/2000	3 mg/ml; 2 µl/CPu	IHC	21.8	
33		Kathy Collier #3	unilat CN 6-OHDA	12/28/2000	3 mg/ml; 2 µl/CPu	IHC	21.8	
34		Margie Ariano #4	unilat CN 6-OHDA	12/28/2000	3 mg/ml; 2 µl/CPu	IHC	21.8	
35								
36	1/10/2001	Kathy Collier #1	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	IHC	20.8	
37		Kathy Collier #2	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	IHC	20.8	
38		Kathy Collier #3	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	IHC	20.8	

	A	B	C	D	E	F	G	H
39		Tim Collier #4	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
40		Tim Collier #5	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
41		Tim Collier #6	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
42		Tim Collier #7	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
43		Tim Collier #8	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
44		Kathy Collier #9	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
45		Tim Collier #10	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
46		Margie Ariano #11	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
47		Tim Collier #12	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
48		Tim Collier #13	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	IHC	20.2	
49		Tim Collier #14	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
50		Kathy Collier #15	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	IHC	20.3	
51		Tim Collier #17	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
52		Tim Collier #18	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
53		Tim Collier #19	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
54		Margie Ariano #22	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
55		Kathy Collier #23	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
56		Tim Collier #24	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 ml/CPu	IHC	20.3	
57		Tim Collier #25	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
58		Kathy Collier #26	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
59		Kathy Collier #27	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
60		Tim Collier #28	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 ml/CPu	IHC	20.3	
61		Tim Collier #29	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
62		Margie Ariano #30	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
63		Kathy Collier #31	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
64		Tim Collier #32	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 µl/CPu	HPLC	Ellsworth 2.21.01	
65		Kathy Collier #33	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 ml/CPu	IHC	20.3	
66		Tim Collier #34	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 ml/CPu	IHC	20.3	
67		Kathy Collier #35	unilat CN 6-OHDA	1/17/2001	3 mg/ml; 2 ml/CPu	IHC	20.3	
68		Tim Collier #36	unilat CN 6-OHDA	2/7/2001	3 mg/ml; 2 ml/CPu	IHC	20.8	
69								
70	3/12/2001	Margie Ariano #1	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.4	
71		Tim Collier #2	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.4	
72		Kathy Collier #3	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.4	
73		Margie Ariano #4	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	
74		Tim Collier #5	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	

Surgical Log

	A	B	C	D	E	F	G	H
75		Kathy Collier #6	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	
76		Margie Ariano #7	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
77		Tim Collier #8	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
78		Kathy Collier #9	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
79		Margie Ariano #10	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	
80		Tim Collier #11	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	
81		Kathy Collier #12	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	24h TMT (A)	Ellsworth	
82		Margie Ariano #13	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
83		Tim Collier #14	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
84		Kathy Collier #15	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
85	no SN	Margie Ariano #16	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
86		Tim Collier #17	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
87		Kathy Collier #18	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	Ellsworth	
88		Margie Ariano #19	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.4	
89		Tim Collier #20	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
90		Kathy Collier #21	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
91		Margie Ariano #22	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
92	no SN	Tim Collier #23	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
93		Kathy Collier #24	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
94		Margie Ariano #25	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
95		Tim Collier #26	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
96		Kathy Collier #27	unilat CN 6-OHDA	4/10/2001	3 mg/ml; 2 µl/CPu	control (B)	Ellsworth	
97		Margie Ariano #28	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
98		Tim Collier #29	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
99		Kathy Collier #30	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
100		Margie Ariano #31	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
101		Kathy Collier #32	unilat CN 6-OHDA	5/14/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.4	
102								
103	5/16/2001	#1 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 ml/CPu	4 wk TMT (A)	HPLC	
104		#2 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 ml/CPu	4 wk TMT (A)	HPLC	
105		#3 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 ml/CPu	4 wk TMT (A)	HPLC	
106		#4 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 ml/CPu	4 wk TMT (A)	HPLC	
107		#5 Kathy Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
108		#6 Tim Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
109		#7 Margie Ariano	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
110		#8 Kathy Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
111		#9 Margie Ariano	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
112		#7 Margie Ariano	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
113		#8 Kathy Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
114		#9 Margie Ariano	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
115		#10 Kathy Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	HPLC	

Surgical Log

	A	B	C	D	E	F	G	H
116		#11 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	HPLC	
117		#12 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	HPLC	
118		#13 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
119		#14 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
120		#15 Kathy Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
121		#16 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
122		#17 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
123		#18 Kathy Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
124		#19 Tim Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
125		#20 Kathy Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
126		#21 Tim Collier	unilat CN 6-OHDA	6/13/2001	3 mg/ml; 2 µl/CPu	4 week lesion	IHC, 21.2	
127		#22 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	HPLC	
128		#23 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	HPLC	
129		#24 Kathy Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	HPLC	
130		#25 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	HPLC	
131		#26 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	HPLC	
132		#27 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.7	
133		#28 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.7	
134		#29 Margie Ariano	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.7	
135		#30 Kathy Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	control (B)	IHC, 20.7	
136		#31 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.5	
137		#32 Tim Collier	unilat CN 6-OHDA	7/13/2001	3 mg/ml; 2 µl/CPu	4 wk TMT (A)	IHC, 20.7	
138								
139								
140								
141		All IHC samples were lost in freezer debacle 12/08/02						

Striatopallidal Changes in a Preclinical Rat Model of Parkinson's Disease

A. E. GRISSELL, T. M. BUCHANAN, AND M. A. ARIANO

Department of Neuroscience, The Chicago Medical School, North Chicago, Illinois 60064, USA

KEYWORDS: Parkinson's disease; dopamine; striatum

INTRODUCTION

Parkinson's disease (PD) is characterized by motor symptoms due to substantial nigrostriatal dopamine (DA) loss (~80%). To investigate the neurochemical alterations that occur prior to motor symptoms, an early preclinical model of PD has been produced. Malonate was infused into one substantia nigra causing a partial, unilateral striatal DA depletion. HPLC analysis detected striatal DA loss (~50%) at 4 weeks. Subtle behavioral changes, indicated by asymmetry of limb use, confirmed a DA imbalance.[1] Morphological analysis of the DA-depleted striatum showed enhanced D2 DA receptor staining, signifying receptor upregulation due to partial DA loss;[2] activated (cleaved) caspase-3, an indication of cellular homeostatic imbalances; and 8-oxoguanine, a marker of oxidative nucleic acid damage. Further, alterations occurred specifically in the striatopallidal projection system, based upon coincident expression of enkephalin as a phenotypic marker of the efferents with the neurochemical markers.

ANIMAL PREPARATION

Twenty-eight male Sprague-Dawley rats (~225 g) were lesioned unilaterally using malonate infusion (3 μmoles in 1 μl) into the right substantia nigra. Rats were killed 4 weeks after malonate infusions.

HPLC ANALYSIS

Striata were dissected, frozen on dry ice, and stored at −86°C until HPLC analysis.[3] Striatal DA content was averaged (±SEM): intact 14.09 ± 2.22 ng/mg protein

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versus DA-depleted 8.01 ± 3.38 ng/mg protein ($P < 0.001$, $N = 14$). DOPAC:DA ratios: intact $0.26 \pm .03$; DA-depleted $0.32 \pm .07$ ($P = .008$).

BEHAVIORAL ASSESSMENT

Rat forelimb use was evaluated as described in Tillerson *et al.*[1] Comparisons between ipsilateral (right side, DA-depleted) or contralateral (left side, intact) forelimb use as a percentage of total movements made were calculated, then averaged.

MORPHOLOGICAL EXAMINATION

Frozen brains ($N = 14$) were cut at 10 μm in the coronal plane, mounted on slides, and fixed by immersion in freshly prepared 4% paraformaldehyde (in PBS) for 5 min. The sections were processed for routine, double-labeled immunofluorescence to determine changes in striatopallidal (enkephalin-positive) striatal neurons on the
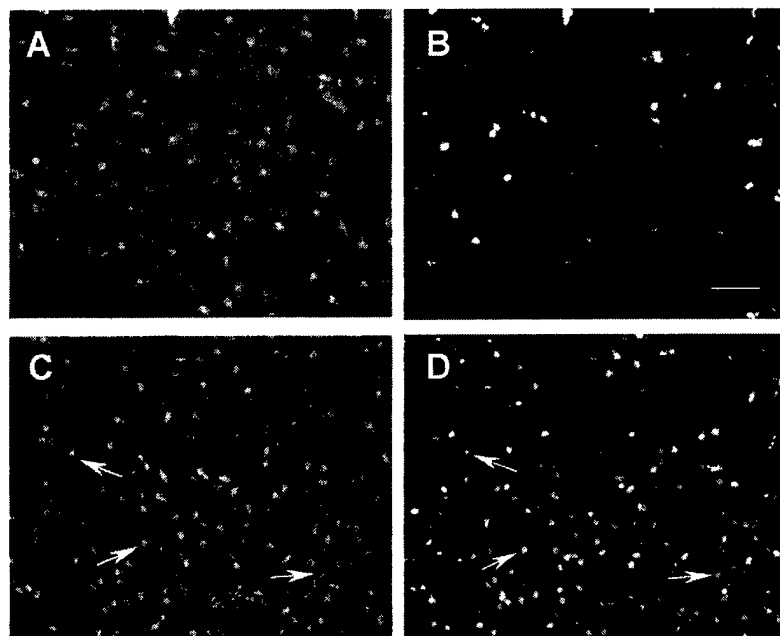
FIGURE 1. Striatopallidal cleaved caspase-3 staining is found in the preclinical PD rat. Enkephalin can identify the projection system and shows the density and shape of medium spiny projection neurons in the intact (**A**) and DA-depleted (**C**) sides. Caspase-3 is cleaved in response to cellular stress and changes in homeostasis produced by DA loss. Cleaved caspase-3 levels were enhanced in DA-depleted (**D**) compared to backgrounds detected in glial cells in intact striatum (**B**). Many striatopallidal neurons express cleaved caspase-3 in the DA-depleted striatum (*arrows* in **C, D**). *Calibration bar* is 100 μm for all images.

intact versus the DA-depleted side. Image acquisition and analysis used methods described previously.[4]

RESULTS

The preclinical PD rat provides a credible model of preclinical stages of PD. Rats have subtle behavior imbalances correlated to the partial striatal DA loss, which showed preferential use of the forepaw ipsilateral to the DA depletion (27.78%, 29.44%, 31.67%) versus the intact side (11.67%, 8.89%, 5.56%) at 2 weeks, 3 weeks, and 4 weeks postinfusion ($N = 9$). Morphological correlates include slight elevation in D2 DA receptor staining and significant ($P < .001$) elevation in expression of cleaved caspase-3 and 8-oxoguanine within the striatopallidal projection pathway ipsilateral to the nigrostriatal DA loss (FIGURE 1), evaluated using cellular luminosity histogram analyses.[4]

CONCLUSIONS

These neurochemical enhancements were unexpected in light of the level (~50%) and duration (4 weeks) of the striatal DA loss. These findings suggest substantial postsynaptic (striatal) changes occur in the early stages of PD and provide a mechanism to evaluate initial neurochemical alterations that may be modified to retard or stop the insidious progression of PD. These results demonstrate the complexity of PD and caution our thinking of the disorder as principally dopaminergic in scope.

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Striatal changes in experimental preclinical Parkinson's disease: Behavioral and neurochemical characterization after partial dopamine depletion

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Abstract

Parkinson's disease (PD) is a prevalent neurological movement disorder that develops following substantial neurodegeneration in the dopamine (DA) containing neurons of the nigrostriatal system. PD demonstrates an early preclinical phase when motor abnormalities develop insidiously. Bradykinesia and difficulty in initiating movements in PD is correlated positively to the depletion of striatal DA levels to nearly 80% of control values. A more thorough study of neurochemical changes in the striatum that occur during the early stages of the disease would elucidate which components of the nigrostriatal system were altered as the DA levels become depleted. We produced a rat model to mimic early, preclinical PD using unilateral intranigral infusion of 6-hydroxydopamine to achieve approximately 50% striatal DA depletion, 4 weeks after neurotoxin placement as assessed using HPLC detection. This partial lesion could be detected by limb-use asymmetries, providing a behavioral indication of the striatal DA imbalance. Striatal neurochemical changes included increased expression of the D2 DA receptor but no consistent changes in D1 DA receptors similar to results reported for larger DA lesions. Mitochondrial membrane potential collapse was indicated by elevated cytochrome C staining. Activated caspase-3 and its actin cleavage product fractin, were increased significantly in the DA depleted striatum, but no cell losses occurred. Elevations were selective for striatopallidal neurons and provide a potential mechanism for the structural changes previously described in DA depleted striatal neurons. The results suggested that striatopallidal homeostatic imbalances may be a feature in preclinical PD that can be used to design novel intervention strategies beyond DA replacement therapy.

KEY WORDS: Parkinson's disease, partial dopamine loss, striatopallidal neurons, cleaved caspase-3, fractin, limb use asymmetry

Introduction

Parkinson's disease (PD) motor symptoms develop following substantial loss of striatal dopamine (DA) terminals (Riederer and Wuketich, 1976). The early disease phase prior to the onset of obvious motor symptoms is termed preclinical PD. Examination of neurochemical changes in the striatum during this time period is lacking and may provide clues to the vulnerability and plasticity of specific postsynaptic components within the nigrostriatal system. Numerous environmental and biochemical compounds can bring about accelerated deterioration of the nigrostriatal pathway (Langston et al, 1983; Rosenberg et al, 1989; Betarbet et al, 2000; McCormick et al, 2002), but those studies employed rapid, near total destruction of the DA neurons. Partial depletion of DA provides an opportunity to model initial phases of PD when DA neurons are undergoing change, but have not degenerated completely (Bergstrom et al, 2001). Consideration of striatal neurochemistry in early PD with respect to identified projection systems may elucidate mechanisms underlying their functional opposition and offer alternative PD therapies that do not rely solely on DA replacement.

DA depletion is the principal neurochemical deficit in PD, and the keystone diagnostic measure is that L-DOPA therapy improves the cardinal signs of the disorder (Factor and Weiner, 2002). Current treatment strategies seek to postpone L-DOPA use as long as possible, impeding its cycle of induced dyskinesias and debilitating side effects (Bezard et al, 2003). PD symptoms arise from the slowly progressing striatal DA depletion and cause differences between the two striatal projection systems such as elevation of D2 DA receptors (Gerfen et al, 1990; Nisenbaum et al, 1996), changes in tertiary spine densities (Arbuthnott et al, 2000), dendritic architecture (Ingham et al, 1989), and augmentation of glutamate responsiveness (Emmi et al, 1996; Calabresi et al, 2000). We produced a preclinical PD rat model using unilateral intranigral 6-OHDA infusions to partially lesion nigrostriatal DA inputs. Unilateral loss of nigrostriatal DA can be monitored by limb-use asymmetry that reflects striatal imbalances (Schallert et al, 2000; Tillerson et al, 2001). We assessed residual striatal DA levels at 4 weeks using HPLC, and then compared cellular changes in equivalent cohorts. This manipulation caused consistent alterations in neurochemical parameters in the DA compromised striatum.

We found that striatal caspase-3 activation occurred and its protein staining levels were elevated in the DA depleted striatum in contrast to the DA intact side. Components in the programmed cell death pathway upstream (cytochrome C) and downstream (fractin) of cleaved caspase-3 also were elevated, indicative of the activation of the intrinsic, mitochondria-driven pathway of caspase activation. These changes were prominent within striatopallidal neurons but not the striatoni-

gral outflow, as determined through simultaneous phenotypic neuropeptide identification of the efferents. Elevations in apoptosis indices occurred in the absence of cell loss, and thus suggested that these events may designate transient homeo-static imbalances contemporaneous with the DA depletion. Taken together the data may elucidate alternative intervention schemes to retard or reduce L-DOPA therapy and define the cellular mechanism underlying functional differences in striatal neurochemical deterioration early in PD.

Methods

Animals.

All animal handling and use were approved by the Institutional Animal Care and Use Committee at the Chicago Medical School and conformed to the *USPHS Guide for the Care and Use of Laboratory Animals*. Male Sprague-Dawley rats (175-200 gm) were obtained from a registered supplier (Harlan, Indianapolis, IN). Animals were prepared as follows: 1) unilateral 6-OHDA (Sigma Chemical Co., St. Louis, MO) infused into the right substantia nigra (1 μl volume; N = 6, HPLC; N = 12, immunohistochemistry; N=3, western analyses), and 2) sham intranigral infusion (1 μl infusion of 0.02% ascorbate in sterile saline, N = 2, immunohistochemistry). Stereotaxic coordinates were AP -5.0mm, L –1.7mm, V –8 mm for the nigral infusion, according to the atlas of Paxinos and Watson (1986).

HPLC.

Whole brains were placed into a brain mold and a coronal cut, just caudal to the hypophysis was made to bisect the brain in half. The rostral portion was further bisected in the sagittal plane and the left and right striata were dissected over ice using the anterior commissure as the rostral boundary. Dissected right and left striata and right and left substantia nigra were stored at -85°C until assayed. DA concentration in tissue samples was measured by HPLC with electrochemical detection, as described by Elsworth et al. (1989) with modifications. Each sample was sonicated in ice-cold 0.1 M perchloric acid, containing EDTA (0.1 mM), and dihydroxyben-zylamine as an internal standard. The homogenate was centrifuged (23,000g, 20 min, 4°C), and the pellet saved for protein determination. A portion of the supernatant was mixed with 3M Tris base and immediately transferred to a small alumina column (10 mg, type WA-4, Sigma, St. Louis, MO), to isolate and concentrate the catechols. After washing the column with water, catechols were eluted with 0.1M oxalic acid. An aliquot was separated on a reverse-phase 3 μm C-18 Microsorb-MV 100 x 4.6 mm column (Varian Inc., Walnut Creek, CA) under isocratic conditions. The mobile phase, delivered at 0.5 ml/min (PM-92E pump, BAS, West Lafayette, IN), contained 30 mM sodium citrate, 15 mM sodium phosphate, 2 mM 1-octane-sulfonate, 0.025 mM EDTA, 6% acetonitrile, 0.6% tetrahydrofuran and 0.1% diethylamine at pH 3.1. The glassy-carbon working electrode, contained in a BAS radial

flowcell, was maintained at a potential of 0.70 volts vs a Ag/AgCl reference electrode. A detection limit of 5-10 fmol DA can be achieved with an LC-4C (BAS) amperometric controller. Quantification was accomplished by dividing the peak height of the DA response by the internal standard, and referring this ratio to external standards. Tissue concentration of DA was corrected for the amount of protein in the sample analyzed. For statistical analyses, comparisons were made within and between treatment groups, with a one-way ANOVA. Significance was set at $p < 0.05$.

Behavioral Analysis.

The severity of nigrostriatal DA damage was evaluated using a sensitive behavioral task that examined limb asymmetries (Schallert and Tillerson, 1999; Tillerson et al, 2001). Forelimb use was assessed in a Plexiglas cylinder (16 X 25 cm) with mirrors placed behind the cylinder to allow 360° viewing of movements, 24-hours following neurotoxin infusions and at 8, 14, 21, and 28 days, once per week for the duration of the experiment. Each rat was videotaped shortly after the dark cycle commenced, using red light illumination. Behaviors were scored using slow-motion playback of the session. Right and left forepaw placement on the vertical surface, as well as simultaneous forepaw (co-use) was scored. Data were collected for the first 20 movements, or 5 min, whichever event came first. Scores were averaged across all animals and the percent usage for each forepaw, and limb co-use was calculated according to specific parameters: 1) Simultaneous placement of the forepaws on the cylinder wall was scored as co-use. 2) Opposing limb contact on the cylinder wall while the initial forelimb placement was maintained also was scored as co-use. 3) Both paws had to be lifted from the cylinder wall surface for a subsequent movement to be scored. 4) The movement was not scored if the forepaw handedness or movement category could not be determined. All animal scores were averaged for a specific time point post-lesion, and are presented as percentage limb use of the total movements made during the taped session. Differences were compared using two-tailed paired *t*-tests and considered significant at $p < 0.05$.

Immunohistochemistry.

Specific polyclonal antisera generated against the DA receptor subtypes have been characterized for D1 (Ariano and Sibley, 1994), D2 (McVittie et al, 1991), D3 (Ariano and Sibley, 1994), D4 (Ariano et al, 1997b), and D5 (Ariano et al, 1997a) DA receptor subtypes. Affinity purified antisera were purchased as follows: cleaved caspase 3 (Cell Signal Technology in So. San Francisco, CA; Chemicon in Temecula, CA; and Zymed in So. San Francisco, CA), cytochrome C (Zymed), enkephalin (Chemicon), fractin (BD Pharmingen, San Diego, CA), substance P (Chemicon) and monoclonal antibodies against tyrosine hydroxylase (TH), NeuN, and enkephalin were obtained from Chemicon. The antisera were diluted in phosphate-buffered saline (PBS, pH 7.2) as follows: D1 @ 1:300, D2 @ 1:200, D3 @ 1:200, D4 @ 1:200, D5 @

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1:200, TH @ 1:600, NeuN @ 1:200, enkephalin @ 1:100, caspase 3 @ 1:100, cytochrome C @ 1:200, fractin @ 1:200, and substance P @ 1:50. Slide-mounted, fresh-frozen coronal tissue sections were employed to detect the DA receptor subtypes because the epitopes for these proteins do not tolerate chemical fixation by perfusion and do not survive cryoprotection treatments. Thus, evaluations of all neurochemical components were determined using this tissue processing approach to conserve on animal numbers, normalize tissue manipulations and obviate shrinkage artifacts. When immunofluorescence detection did not use the DA receptor subtypes, or was performed as a double labeling experiment to evaluate phenotypic identification of striatal projection systems, slide mounted sections were immersion fixed for 5 min in cold, freshly prepared 4% paraformaldehyde in PBS.

The primary antisera were applied to the sections and incubated overnight at 4°C in a humidified environment. The next day, unbound primary antisera were rinsed off and fluorescently labeled secondary antisera (donkey anti -rabbit, -mouse or -guinea pig conjugated with either Cy2 or Cy3, Jackson ImmunoResearch, West Grove, PA) were applied, and diluted in PBS (1:200) for 2 h at 4°C in a humidified environment. Controls included: 1) use of multiple antisera, directed against different epitopes of the DA receptor protein sequences, or commercial reagents obtained from different vendors, 2) use of preimmune sera when available, 3) omission of the primary antisera, and 4) adsorption challenge of the primary antisera with the control antigen when possible. No differences in staining distribution or patterns were noted with antisera directed against different DA receptor epitopes, or with commercial antisera from different vendors or different manufactured lots. Other controls showed no fluorescence staining in the tissue sections.

Microscopy.

Tissues were frozen in powdered dry ice, mounted in the coronal plane, and sectioned on a cryostat (10 μm). Following immunofluorescence processing, sections were examined using a fluorescence microscope (Olympus BX41). Yellow-green fluorescence was excited at 488 nm and detected using 515-540 nm band pass filter. Red fluorescence was excited at 543 nm and visualized with a 570 nm long pass filter. Digitized images for intact and DA depleted sides were matched to similar regions in the dorsal striatum. Image acquisition and exposure settings were identical for each antiserum detected in an individual section and the exposure settings were optimized for images obtained from the intact side, thus normalizing subsequent DA depleted striatal data to the control side. Image acquisition followed a specific exposure sequence in the coronal tissue sections, from dorsomedial, dorsocentral, dorsolateral. Experimental runs were performed at least twice in each experimental brain for each antibody. The sections were examined immediately upon completion of the incubations.

Data analysis.

Twelve images were obtained from 3 different coronal sections mounted onto one glass slide and stained for one particular antigen. The rodent striatum is penetrated by myelinated fiber bundles of the internal capsule. To minimize contributions from this unstained compartment in the neuropil stain seen using TH, a square of pixel dimensions 150 X 150 was placed over three distinct striatal neuropil sites in each image, to eliminate inclusion of the fiber bundle values in the luminosity histograms. Conversion of fluorescent staining intensity into histogram luminosity values assigned a numerical value to the averaged grayscale level of the staining intensities (range 0-255) within the pixel square. Values are reported as luminosity means ± standard error (se), and upper case Ns are the number of rats sampled, while lower case n denotes the number of cells evaluated. For statistical comparisons, paired t-tests (two-tailed) were performed on the differences in average median luminosity values from regionally matched intact and DA depleted sections. Differences were considered statistically significant when $p < 0.001$.

Cellular luminosity histograms were obtained from individual striatal neurons in staining runs performed on the intact and DA depleted side for antigens having somata reactivity. The background luminosity in an experiment was determined using the average values of three similarly sized pixel circles (40 px X 40 px) corresponding to the medium-sized striatal neuron cell body diameter within the internal capsule fiber bundles penetrating the striatum. Neurons were chosen to be $\geq 20\%$ of the averaged background luminosity signal and intersected a counting grid. The counting grid was overlaid on top of acquired images, and any neuron that intersected the vertical lines of the counting grid was circumscribed using a marquee at a fixed size to outline the medium-sized neurons (40 pixels X 40 pixels using a 20X objective). The counting grid contained vertical lines spaced at 0.5 inch intervals. Data are reported as individual cellular fluorescence staining intensity (luminosity) of the antigens within the DA depleted somata populations, divided by the signal measured in the intact striatal perikarya sample. These results are reported as the averaged luminosity values ± se for all the counted neurons for the immunofluorescent data set minus the background luminosity signal, in an individual antibody run. Statistical differences were compared using paired t-tests (two-tailed) on the averaged median values, and were considered statistically significant at $p < 0.001$.

Western analysis.

Brains were removed rapidly (N = 3). The striatum was isolated, frozen immediately on dry ice, and stored at -85°C until processed. Samples were homogenized by sonication in 20 vol of buffer (w:v), containing 20 mM Tris pH 7.4, 0.1% SDS, with Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA; homogenizing buffer). Samples were incubated in this buffer for 30 minutes at 4°C, and insoluble material was removed by centrifugation at 15,000 X g for 30 min. Protein concentrations were assayed by the method of Bradford, using a Bio-Rad kit (Hercules, CA). Samples of equivalent protein concentration from the intact and DA depleted striatal homogenates were separated by SDS-PAGE on 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA), then transferred to PVDF membranes (Millipore, Billerica, MA). Blots were

incubated with primary antisera generated against cleaved caspase 3 (Chemicon) or fractin (BD Pharmigen, San Diego, CA) in buffer containing 5% nonfat dairy milk overnight with agitation at room temperature. Secondary, peroxidase-conjugated antisera (Jackson ImmunoResearch) were used to detect the antisera on the blot. Bands were detected using ECL detection reagent (Amersham, Inc., Arlington Heights, IL), and differences in signals were determined. Data is presented as the percent change of the DA lesioned striatum compared to the intact side, and were evaluated using paired two tailed t-tests with differences considered statistically different @ $p < 0.05$. Molecular weights were determined on the basis of the mobility of prestained SDS-PAGE standards, broad range (Bio-Rad).

Results

Partial striatal DA loss was monitored by HPLC and limb use asymmetry

Initial experiments established the appropriate dosage of neurotoxin and infusion volume that would render ~50% loss of striatal DA at 4 weeks following nigrostriatal interruption. HPLC was used to evaluate residual striatal DA levels and established parameters for 6-OHDA The infusions of 6-OHDA (6 μgm in 1 μl volume; N=10) yielded 58% loss in the DA depleted striatum @ 4 weeks. All subsequent neurotoxin lesions were made using this 6-OHDA concentration. A unilateral partial DA depletion model provided a within-animal control for changes on the side contralateral to the neurotoxin infusion.

At this unilateral DA depletion level (range of 37-65% depletion), only subtle behavioral effects can be elicited by DA receptor stimulation (Barneoud et al, 1995; Emmi et al, 1996; Kirik et al, 1998). We sought alternative ways to evaluate DA loss that would not confound morphological DA receptor distributions by a "priming effect" following receptor stimulation (Morelli and Di Chiara, 1987; Ariano et al, 1997; Dumartin et al, 1998), and would determine *a priori* the extent of the striatal DA imbalance through behavioral indices. Limb-use asymmetries have been characterized extensively (Schallert and Tillerson, 1999; Schallert et al, 2000), and have been employed to determine striatal DA losses that reflect 30-85% depletions. Rats were examined prior to the interruption of the nigrostriatal pathway through videotape recording of their vertical exploration in a Plexiglas cylinder to determine if a preferred handedness existed. Rats did not show a preferential paw use and thus all neurotoxin infusion sites were located within the right nigrostriatal system.

The limb use asymmetry method and scoring procedure described by Tillerson (et al, 2001) were employed to evaluate striatal DA imbalances at weekly intervals following intranigral neurotoxin treatments (Fig 1). Rats consistently preferred the use of the forepaw ipsilateral (diagonal barred histogram) to the striatal DA depletion, in agreement with the findings of the Schallert laboratory (Schallert and Tillerson, 1999; Schallert et al, 2000; Tillerson, et al, 2001). The most robust disparity in the use of the forepaw ipsilateral to the DA

depletion occurred at 24 hours, and most likely reflected an acute response to manipulation of the nigrostriatal afferents that caused a transient striatal DA loss near 75%. The calculation of DA loss was based upon subtraction of % contralateral use from % ipsilateral use, and used the graphic relationship described by Schallert and colleagues (*www.schallertlab.org*). This ipsilateral forepaw usage pattern stabilized between 2-3 weeks (~30% of total movements), but was always more prevalent than that scored for the forelimb contralateral to the neurotoxin treatment (black histogram; < 10% of total movements). This corresponded to 44% residual striatal DA after intranigral 6-OHDA infusion, compared to the intact side. Co-use of the forepaws is plotted as white histograms (Fig 1).

Morphological correlates of partial nigrostriatal interruption

Staining for TH, the rate-limiting enzyme in DA synthesis was used as a morphological correlate to the behavioral limb use asymmetry results to determine residual nigrostriatal DA terminals, 4 weeks after 6-OHDA the infusions. TH immunofluorescence was reduced significantly within the neuropil of the DA depleted striatum, compared to the intact side (Fig. 2A). The decrease in TH staining averaged -41% (range of 34 to 65% loss, N = 12) in the DA depleted striatum contrasted to the intact side. Sham animals were infused with ascorbate only, and showed no differences in TH immunofluorescence luminosity signals between striata (mean of 101% DA depleted versus intact, N = 2, data not shown).

Toluidine blue was used to establish if cell losses occurred and no changes were noted in either the density or morphology of cells within the intact or DA depleted striatum (Fig 2B). Cell counts, generated from one rat were made from 6 striatal images obtained for the control side (n = 373 cells), and 6 images from the DA depleted side (n = 364 cells), 4 weeks after intranigral infusion of 6-OHDA, and the results showed no difference in density. To specifically assess whether neuronal losses occurred, the selective neuronal protein marker, NeuN was examined. Immunofluorescent detection of NeuN (Fig 2C) further validated that the density and cytoarchitecture of striatal neurons were not affected by the neurotoxin treatment. The number of NeuN stained somata detected within the intact striatum was 4,556 versus 4,604 sampled within the DA depleted striata (N = 12 rats, 6 image sets per rat).

Striatal expression of DA receptor subtypes

A striatal characteristic after DA loss is the elevation of the D2 DA receptor, and has been monitored using receptor binding assays (Cadet et al, 1991; Fornaretto et al, 1993; Narang and Wamsley, 1995; Piggott et al, 1999), mRNA transcript expression (Gerfen et al, 1990; Xu et al, 1992; Fornaretto et al, 1993; Narang and Wamsley, 1995) and results from the release of negative modulation of the D2 DA receptor gene (Minowa et al, 1994; Hwang et al, 2001). Changes in other DA receptors, which exhibit substantial expression levels within the striatum (D1 and D3) have proved inconsistent (Ariano, 1988; 1989; Gerfen et al, 1990; Cadet et al, 1991;

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Fornaretto et al, 1993; Narang and Wamsley, 1995). Those studies employed near complete lesions of the nigrostriatal afferents, and thus it was necessary to assess if these characteristics were detected following partial DA depletion of the striatum. Cellular luminosity signals for D1, D2, D3, D4 and D5 DA receptor protein staining were evaluated using subtype specific antisera (McVittie et al, 1991; Ariano and Sibley, 1994; Ariano et al, 1997a; 1997b), and compared between the intact and DA depleted striata. D1 DA receptor staining was not changed significantly in staining levels in the partially denervated striata compared to the intact side. By contrast, D2 and D3 DA receptor signals were elevated consistently within the experimental PD rats, ranging from +11% to +40%, and the cellular luminosity values achieved statistical significance (Table I). Immunofluorescent staining for D4 and D5 DA receptors did not provide consistent results and was not studied further.

The morphological staining pattern for striatal D2 DA receptor proteins was located predominantly in medium-diameter striatal neurons, within a thin rim of reactive cytoplasm surrounding a central nucleus (Fig 3). Partial DA depletion induced the level of D2 DA receptor fluorescence (+20% in this experiment, $p < 0.001$, Fig 3A versus 3C). Higher magnification examination of the D2 receptor expression showed punctate "hot spots" of staining within the cytoplasm of the medium sized neurons near the membrane perimeter, which were larger in size and more intense in fluorescent signal following partial loss of DA compared to the intact side (arrows, Fig 3B versus 3D).

Cleaved caspase 3 was elevated in DA depleted striatal neurons

We detected significant elevations in the number of striatal neurons stained for cleaved caspase 3 in DA depleted striata, 4 weeks following the 6-OHDA infusions compared to the intact side (Fig 4A). Neurons were distinguished by the presence of NeuN (green) and co-localization with cleaved caspase 3 (red) staining was detected as yellow somata (arrows). The intact striatum exhibited sparse expression of neuronal cleaved caspase 3, however non-neuronal (probably glia) cell bodies were evident as intense red structures throughout the striatum and occasional reactive neurons could be detected (arrow). Partial DA depletion produced extensive increases in the number of cleaved caspase 3 stained neurons (arrows), and fluorescence intensity measurements demonstrated +40% increase in somata signal luminosity; values for the representative experiment illustrated in Fig 4A were intact side @ 48.6 ± 1.7 versus DA depleted striatum @ 68.6 ± 1.6, $p < 0.001$. A total of 789 caspase-positive neurons were scored from the intact striatum, and 1,811 were evaluated from the DA depleted side. This was verified in 10 different rats. At one week following 6-OHDA treatment, no elevation in cleaved caspase 3 staining was detected in comparison to the intact side, and these changes also were not detected in the sham operated animals (data not shown).

Cleaved caspase 3 levels were evaluated further in striatal homogenates in another series of rats (N = 3) by western blot analyses (Fig 4B) after intranigral infusion of 6-OHDA.

These studies used the same antibody reagent as was applied to the cryostat tissue sections to ascertain the morphological distributions of the activated enzyme. The findings demonstrated that cleaved caspase 3 was present in the intact striatum, but was elevated robustly after partial loss of DA. The DA depletion resulted in striatal cleaved caspase 3 levels (rat #1, +290%; rat #2, +590%; rat #3, +308%).

Activated caspase 3 was expressed preferentially in striatopallidal projection neurons after DA depletion

Models of functional basal ganglia circuitry in PD argue that the striatopallidal pathway is preferentially affected by the loss of nigrostriatal DA (Gerfen et al, 1990; Calabresi et al, 2000; Graybiel, 2000; Beal, 2001; Breysse et al, 2003). We reasoned that imbalances in cellular homeostasis should be expressed readily within the striatopallidal pathway but be less pronounced within the striatonigral system. We examined the co-localization of cleaved caspase 3 (red label) contrasted with phenotypic identification of the striatopallidal projection neurons using enkephalin staining (seen as a green signal, Fig 5A), while the striatonigral somata were distinguished by substance P expression (likewise visible using a green signal Fig 5B; Aizman et al, 2000; Graybiel 2000). As we predicted, striatopallidal co-expression of cleaved caspase 3 (yellow somata, arrows, Fig 5A) was elevated significantly in DA depleted striatum contrasted to the intact side (+40%, cellular luminosity values for intact 90 ± 3.4 versus DA depleted 127 ± 4 @ $p < 0.001$), but was not enhanced within the striatonigral system (Fig 5B). Cellular luminosity values for striatonigral cleaved caspase 3 were intact 100 ± 2 contrasted to the DA depleted values 111 ± 5 @ $p = 0.02$, and thus the +11% did not meet the criterion for significance. A total of 739 striatopallidal caspase 3 stained neurons were evaluated from the intact striatum, and 931 were scored from the DA depleted side. Caspase 3 stained striatonigral neuron results were obtained from 631 scored projection cells on the intact striatum and 587 from the DA depleted side. This finding was replicated in 10 6-OHDA lesioned rats.

Mitochondrial stress indicators were elevated in DA depleted striatopallidal neurons

If caspase 3 cleavage was initiated via mitochondrial mechanisms after partial DA depletion, we would detect enhancements in staining of components of the apoptosome (Kroemer and Reed, 2000). These elevations should be more prominent in the striatopallidal system in contrast to that seen in the striatonigral projection system, based upon our cleaved caspase 3 data. Apaf-1 (apoptosis protease-activating factor 1) is a constitutive cytoplasmic protein, associates with cytochrome C and cleaved caspase-9 to form the apoptosome (Yoshida et al, 1998; Zou et al, 1997), and is indicative of mitochondrial energy insufficiency, causing collapse of the membrane potential in this organelle. A second function of the apoptosome is the cleavage of the zymogen of caspase 3 to its active form, which provides an upstream indicator of activation of the caspase 3 cleavage cascade. We examined the distribution of cytochrome C in the partially DA depleted rats,

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since defects and changes in mitochondria functioning have been reported to be mechanisms underlying changes in substantia nigra DA neurons in spontaneous PD (Langston et al, 1983; Nicklas and Heikkila, 1985; Rosenberg et al, 1989; Greenamyre et al, 1999; Sherer et al, 2002).

Cytochrome C (red label) was detected immunohistochemically (Fig 6) in rats subjected to unilateral intranigral 6-OHDA, within medium-diameter striatal neurons as a thin, diffuse rim of cytoplasmic signal (insets in Fig 6), and throughout the neuropil. Fiber bundles of the internal capsule were not reactive for the enzyme. Striatal projection systems were identified phenotypically, as described above, and were distinguished by green labeling throughout the somata of reactive cells. Co-expression of cytochrome C within either projection system was evident (arrows, Fig 6), however only the striatopallidal neurons within the DA depleted striatum demonstrated elevations of cytochrome C. This caused the image to appear redder by comparison with the other three panels in Fig 6, and argued for selective formation of the apoptosome and functional instability of the mitochondria in the striatopallidal neurons deprived of DA. Cellular luminosity values for this representative experiment were: striatopallidal intact @ 61.5 ± 1.1 versus DA depleted @ 90.7 ± 1.9, $p < 0.001$, showing +47% elevation of the fluorescent signal; striatonigral intact @ 83.2 ± 1.2 and DA depleted @ 83.1 ± 1.7, $p = .99$, and showed no change in staining intensity between the two sides. This experiment was repeated in two other rats treated with intranigral 6-OHDA. The total numbers of medium spiny neurons evaluated for cytochrome C were: intact striatopallidal = 874, DA depleted striatopallidal = 946, intact striatonigral = 800 and DA depleted striatonigral = 704. This outcome was seen in 6 other 6-OHDA lesioned rats.

Actin breakdown was elevated in DA depleted striatopallidal neurons

Actin is cleaved by activated caspase 3 to form fractin (Oo et al, 2002). The appearance of fractin within neurons showed that cleaved caspase 3 was enzymatically active in addition to enhancements in the level of the protein staining which were detected as increased cellular luminosity signals (Fig 4 and 5). Rearrangements in the neuronal cytoskeleton would result from actin cleavage, and numerous studies have reported that DA loss profoundly changes the cytoarchitecture of striatal medium sized projection neurons (c.f., Arbuthnott et al, 2000; Ingham et al, 1989). We evaluated fractin in the partially DA depleted striatum compared to the intact side. Initial studies were combined with NeuN showed that significant elevations of fractin occurred within neurons (data not shown).

We next examined if there was a preferential expression of fractin within the striatopallidal neurons of the DA depleted striatum compared to the intact side, and contrasted the cellular luminosities to experiments performed on the striatonigral system (Fig 7A). Fractin (red signal) was present in the intact striata, and distributed heterogeneously within the cytoplasm of medium sized projection neurons (green signal and shown by arrows) and throughout their processes in the neuropil for both systems; fiber bundles of the internal capsule were not reactive. After partial DA depletion, the staining of fractin was elevated significantly, but only in the striatopallidal neurons. This experiment showed exclusive enhancement of the fractin signal within the striatopallidal neurons of the DA depleted side in contrast to the expression levels in the intact side, or within the striatonigral system. Cellular luminosities for the representative experiment illustrated in Fig 7A were: striatopallidal intact @ 51 ± 1 versus DA depleted @ 76 ± 1.1, $p < 0.001$, +49% elevation; striatonigral intact @ 44 ± 1 versus DA depleted @ 44.2 ± 1, $p = .97$, no change in signal. Morphologically analyzed projection neuron totals stained for fractin were: intact striatopallidal = 714, DA depleted striatopallidal = 818, intact striatonigral = 578 and DA depleted striatonigral = 557. This finding was repeated in 7 other DA depleted rats. Western analyses were used to further verify the quantitative differences in the expression of fractin in whole striatal homogenates following intranigral 6-OHDA (Fig 7B). The blot used to assess caspase 3 levels (Fig 4B) was stripped, and re-probed for fractin using the same antisera reagents employed in the morphological studies. In two of the paired striatal samples, the densitometry differences were +220% in rat #1, and +356% in rat #2, clearly showing enhanced striatal expression of the fractin protein after partial DA depletion.

Discussion

The present studies used a partial, unilateral DA interruption in the nigrostriatal system to define early neurochemical alterations within the striatum that may be triggers for the insidious disease sequelae. Our results demonstrated that the mild to moderate striatal DA imbalance could be monitored behaviorally, and resulted in heightened D2 DA receptor levels, as reported previously in various model systems that have used more massive nigrostriatal interventions (Gerfen et al, 1990; Fornaretto et al, 1993; Minowa et al, 1994) or within postmortem PD striatal tissues (Piggott et al, 1999). What was very remarkable was that even mild striatal DA loss had profound and differential effect on the two projection systems that would modify the responsiveness of the basal ganglia circuits. Surprisingly, elements of the intrinsic programmed cell death pathway, which may be mobilized through perturbations of the mitochondrial membrane potential (Finkel, 2001) were enhanced significantly and selectively in the striatopallidal projection system. Substantial elevations in the protein staining and enzymatic activity of cleaved caspase 3 and constituents upstream (cytochrome C) and downstream (fractin) in this biochemical pathway were detected in our unilateral rat model of early stage PD. These findings argued that enzymatic recruitment of the intrinsic apoptosis cascade occurred as an early striatal event in this PD model, well before the overt motor symptoms characteristic of the disorder. The established role for the activation of caspase 3 is execution of programmed death in severely perturbed cells (Thornberry and Lazebnik, 1998), and cleavage of the inactive zymogen and appearance of the activated form of caspase 3 have been elicited in experimental models of PD *in vitro* and

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in vivo, and have been implicated as a mechanism in nigral DA neurodegeneration in human PD tissues (He et al, 2000; Lu et al, 2000; Mattson, 2000; Qin et al, 2000; Tatton, 2000; Blum et al, 2001; Lu et al, 2001; Turmel et al, 2001; Hartmann et al, 2002; Marti et al, 2002; Nagatsu, 2002). The fact that our studies also detected the robust elevation of activated caspase 3 in the nigrostriatal termination region was unexpected and novel, and lead us to the conjecture that caspase 3 underlies another crucial function beyond launching the programmed cell death sequence early in the PD disease process.

Indeed, activated caspase 3 has alternate cellular roles such as actin breakdown (Oo et al, 2002), cleavage of the tau protein in Alzheimer's disease (Gamblin et al, 2003), and activation of the DNA damage sensing enzyme, poly-ADP ribose polymerase (PARP) in MPTP rodent models of PD (Wang et al, 2003). Recent studies have demonstrated further that a sub lethal anoxic insult to the parietal cortex as well as in a parallel *in vitro* cortical culture system, caused induction of tolerance to subsequent NMDA-mediated excitotoxic stressors through the activation of caspase 3 (Garnier et al, 2003; McLaughlin, et al, 2003). In these latter studies, the appearance of cleaved caspase 3 occurred within a precise chronology following the acute insult, generated reactive oxygen species via protein synthesis, and required opening of the K_{ATP} channel which is embedded in the inner mitochondrial membrane. These findings suggested that activation of the intrinsic apoptosis pathway had occurred and was the cellular mechanism underlying the acute and transient response of cortical neurons to this mild anoxic episode. Interestingly, these changes occurred without the loss of neurons and the authors posited that a neuroprotective role was a realistic alternative for the appearance of cellular constituents normally associated with mitochondrially driven apoptosis (McLaughlin et al, 2003). Garnier and colleagues (2003) extended that report and showed that caspase 3 cleavage of PARP-1 was the specific biochemical event leading to the resistance of cortical neurons to subsequent ischemia.

Those findings, taken together with the results we detected in early stages of DA depletion in the current studies of early stage PD, strongly support an alternative neurochemical function that may be assumed by the programmed cell death pathway within striatal neurons after DA loss from their neurochemical milieu. We would argue that the appearance of cleaved caspase 3 did not signify imminent striatal neurodegeneration, but instead signaled postsynaptic homeostatic imbalances occurred as a result of partial interruption in the nigrostriatal system. Moreover, we evaluated caspase 3 levels in the context of neuronal expression, and specifically in regard to the phenotypically identified projection systems of the striatum. The data clearly showed selective elevation in cellular luminosities for the caspase 3 cascade within the striatopallidal system; these elevations were corroborated further using quantitative western blot analyses. Since we did not detect neuronal losses in the DA-depleted striatum, we hypothesize that the appearance of cleaved caspase 3 may be an early and transient event and subserve an alternative cellular function besides executing apoptosis.

Nigrostriatal DA depletion is a substantial insult to the postsynaptic neurons of the striatum, and while not immedi-

ately lethal, its loss altered striatal somata and dendritic morphology in medium spiny projection neurons (McNeill et al, 1988; Arbuthnott et al, 2000), produced changes in spine densities (Ingham et al 1989), in addition to changing the responsiveness of the basal ganglia circuitry (Calabresi et al, 1993; 2000; Breysse et al, 2003). The breakdown of actin by activated caspase 3 would contribute to re-shaping the cytoarchitecture of the striatal neurons, and would be a direct consequence of mobilization of an alternative action of the intrinsic apoptosis cascade. This was demonstrated in the present work by the elevated staining and enhanced protein expression of fractin in the striatopallidal neurons (Fig 7). Deterioration of the structural components in the striatal projection systems may be precipitated by changes in intracellular homeostasis that initiate elevations in cellular housekeeping enzymes and energy status, further exacerbating the neurochemical changes.

Numerous investigations have demonstrated enhanced apoptosis in PD animal models (He et al, 2000; Mochizuki et al, 2001; Turmel et al, 2001; Bilsland et al, 2002; Marti et al, 2002; Wang et al, 2003) and in human PD postmortem tissues (Hartmann et al, 2000; Nagatsu 2002). These findings strongly suggest that therapeutic benefits may be derived from the use of anti-apoptotic drugs in PD and even used as a co-therapy with routine pharmacologic treatments (Fiskum et al, 2003; Nomoto 2003; Waldmeier 2003) to diminish the expression of L-dopa induced dyskinesias (Bezard et al, 2001). The findings seen in the striatum at early stages of PD add further impetus for the use of inhibitors of apoptosis as part of the drug treatment regimen to alleviate the symptoms of the disease, and increase the life cycle of the targeted neurons.

The disparity in the staining responses which we detected for cytochrome C, cleaved caspase 3 and fractin between the striatonigral and striatopallidal neurons strongly argued for functional differences between the striatal projections at early stages of PD, even before overt movement abnormalities would be detected. Models of basal ganglia function, derived from experimental and imaging studies support a variety of scenarios for the activity patterns through the striatal outflow pathways in PD (Calabresi et al, 2000; Gerfen et al, 1990; Graybiel 2000; Beal 2001), but strongly favor increased responsiveness and an alteration in the firing pattern of neurons in the subthalamic nucleus, a component of the indirect pathway, downstream from the striatopallidal neurons. It is apparent that some neurons comprising the striatopallidal system are particularly sensitive to degeneration via apoptosis in response to manipulation of DA and/or corticosteroid levels (Mitchell et al, 1999). In addition, striatopallidal neurons are preferentially targeted in Huntington's disease, another basal ganglia movement disorder. Although Huntington's disease displays autosomal dominant inheritance and results from the expansion of a polyglutamine sequence within the mutated protein huntingtin, a number of parallels exist between these two basal ganglia movement disorders, including insidious deterioration of neurons due to excitotoxicity (DiFiglia 1990; Albin and Greenamyre, 1992; Aronin et al, 1999; Zeron et al, 2001) and aberrant oxidative energy production (Coyle and Puttfarkin, 1993; Beal, 1995; Ferrante et al, 2000), development of cellular protein inclusions (DiFiglia et al, 1997), and

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changes in striatal neurochemistry that affect many of the same neurotransmitter systems (Cha et al, 1998; Ariano et al, 2002). However what is lacking ultimately is some determination of the special vulnerability inherent in the striatopallidal neurons that makes them particularly at risk following disturbances in the striatal neurochemical environment. Elucidation of the special character of the striatopallidal neurons may contribute to better treatment strategies for both of these basal ganglia movement disorders, and the studies reported here have shown that the disparity exists in neurochemical responsiveness in striatal medium spiny neurons.

Acknowledgements

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Table I. DA Receptor Cellular Luminosities

Experiment	D1	D2	D3
	71 ± 1.5	47 ± 1.3	30 ± 1
Intranigral 6-OHDA[1]	74 ± 1.4 (+5%)	66 ± 1.3 (+40%)*	34 ± 1 (+11%)*

The top number in each column represents staining luminosity in neurons in the intact striatum, while the bottom line is the DA depleted striatum from the same immunofluorescent experiment. Numbers in parentheses denote the %age change in luminosity of the DA depleted striatum. Asterisks show that statistical significance was achieved at $p < 0.001$ between the DA intact and depleted striata for the receptor subtype.

[1]Representative experiment based upon N = 10 rats (~2,300 striatal neurons evaluated for the DA condition for each of the three DA receptor subtypes).
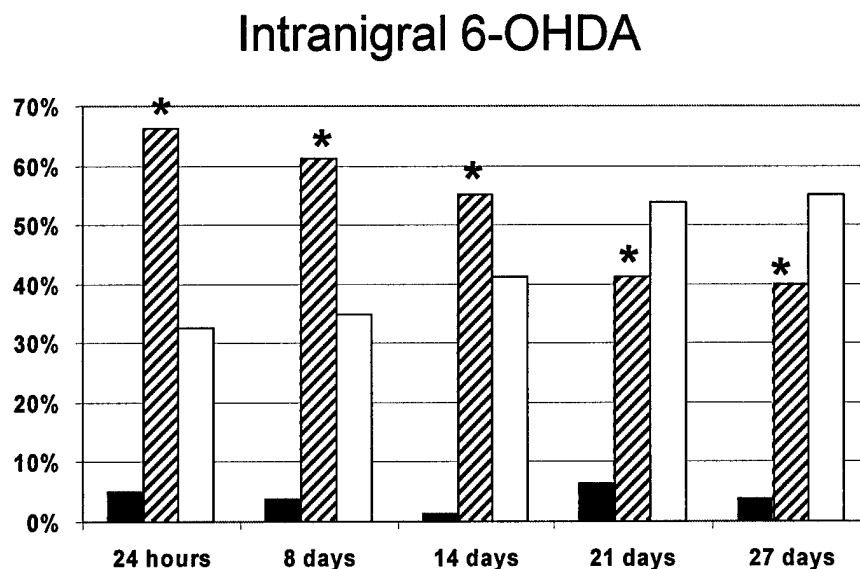
Figure 1

Intranigral 6-OHDA

Figure 1. Behavioral limb asymmetries were evaluated 24 hours, then weekly during the study as described in the text. Fore limb usage was scored after unilateral intranigral infusion of 6-OHDA (6 μgm in 1 μl; N = 8). Scores were averaged across animals at each time point, and the percent co-use (white histogram), ipsilateral to the lesion (DA depleted, diagonal bar histogram) or contralateral to the lesion (DA intact, black histogram) as a percentage of total movements was plotted. Use of the ipsilateral forepaw (DA depleted side) was statistically significant (asterisks) in comparison to the contralateral (DA intact) side ($p < 0.05$).

Figure 2

Intact   **DA depleted**
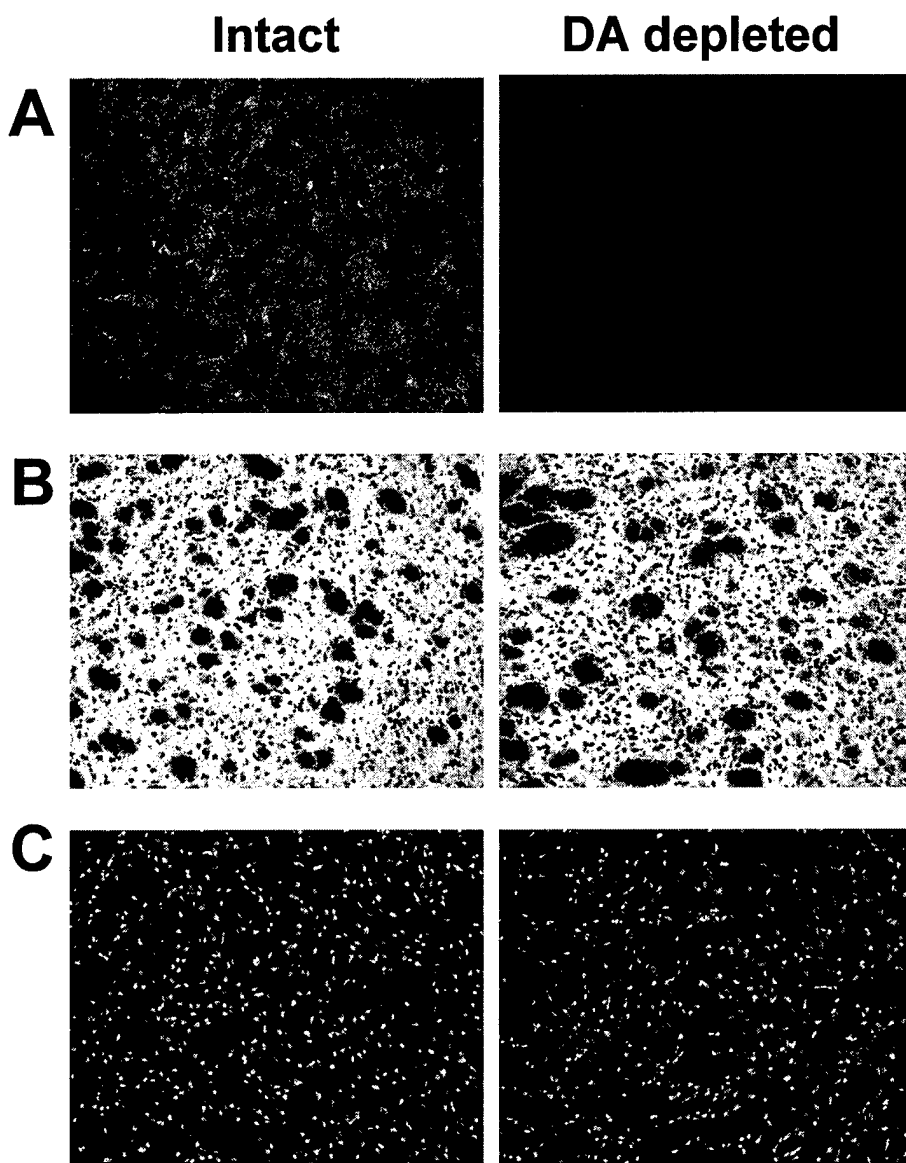
Figure 2. Histological verification of striatal morphology was assessed. *A*, Tyrosine hydroxlase (TH) antisera was applied to striatal sections to detect residual nigrostriatal DA terminals following 6-OHDA. The DA depleted side showed -59% decrease in neuropil staining luminosity compared to the intact striatum in this representative experiment. *B*, Toluidine blue was applied to cryostat sections and cells were counted. No loss in cell number occurred following partial DA depletion. Myelinated elements within the fiber bundles of the internal capsule also attract the dye mordant and appear as black ovoids. *C*, NeuN was used to verify neuronal counts in the intact and DA depleted striata. No losses in density occurred between the striata. Calibration bar applies to all images.
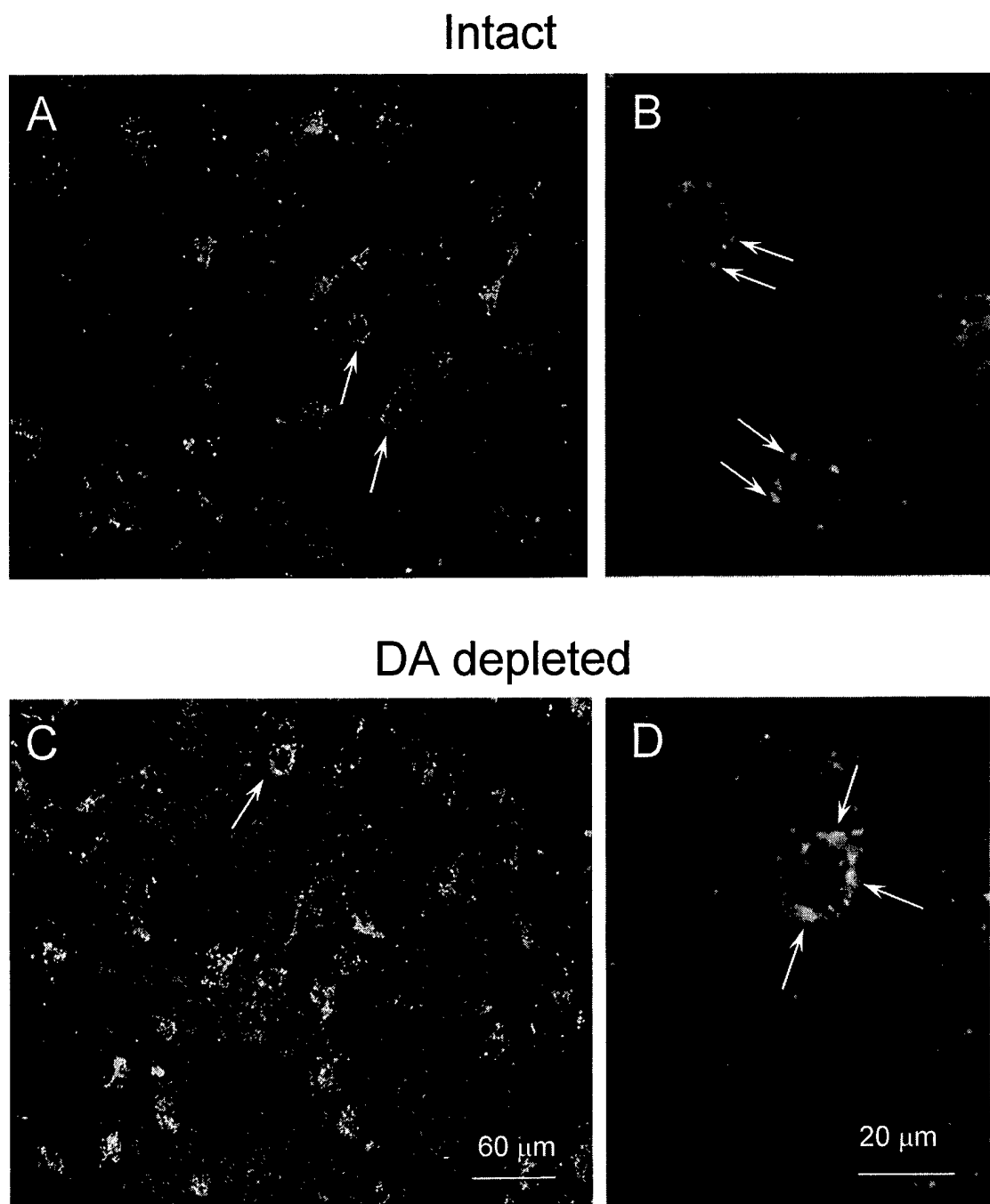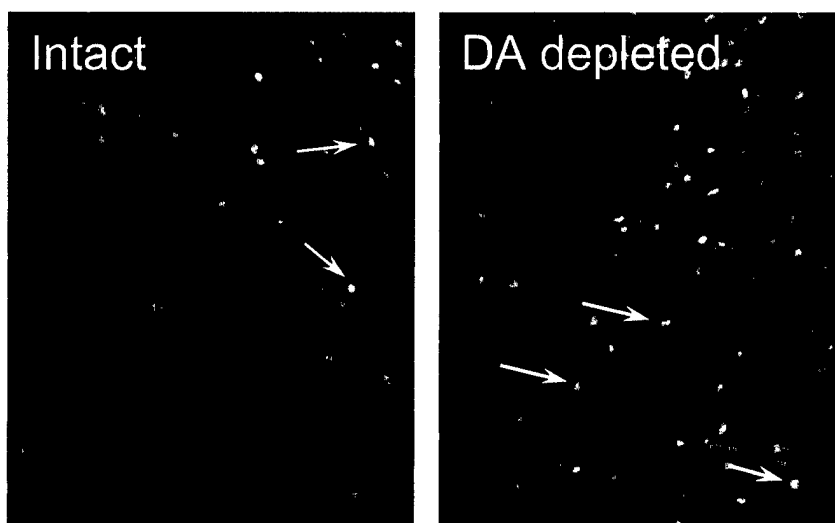
Figure 3

Intact

DA depleted

Figure 3. Striatal D2 DA receptor protein staining was assessed after 6-OHDA infusion produced partial DA depletion. *A*, D2 staining was visible within a group of medium sized neurons (arrows) of the intact striatum and in the neuropil. *B*, At higher magnification, D2 receptor staining was visible as punctate "hot spots" at the somata membrane surface. *C*, Partial DA depletion caused elevation of the fluorescent D2 DA receptor protein signal and at higher magnification (*D*) the punctate spots were more intense in signal and their dimensions had increased. Calibration bars are equivalent for *A,C* and for *B,D*.

Figure 5

A Striatopallidal Cleaved Caspase 3
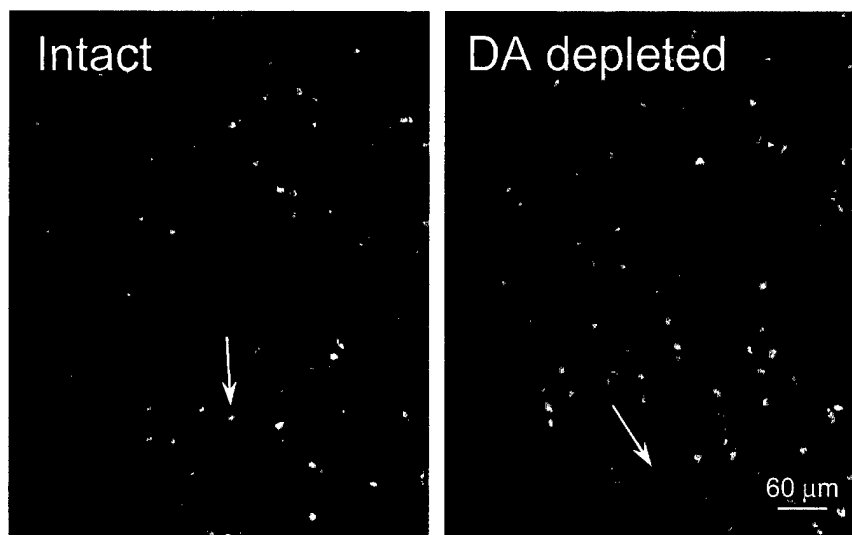

B Striatonigral Cleaved Caspase 3

Figure 5. Cleaved caspase 3 (red) staining was determined within the striatal projection systems using co-localization of neuropeptides (green label) following partial DA depletion. *A*, Striatopallidal cleaved caspase 3 was enhanced significantly within the DA depleted striatum (+40% @ p < 0.001; yellow cells) compared to the intact side. *B*, Staining intensity for striatonigral cleaved caspase 3 staining was not different between the intact or DA depleted striata in this same animal. Calibration bar applies to all images.

Figure 6

A   Striatopallidal Cytochrome C
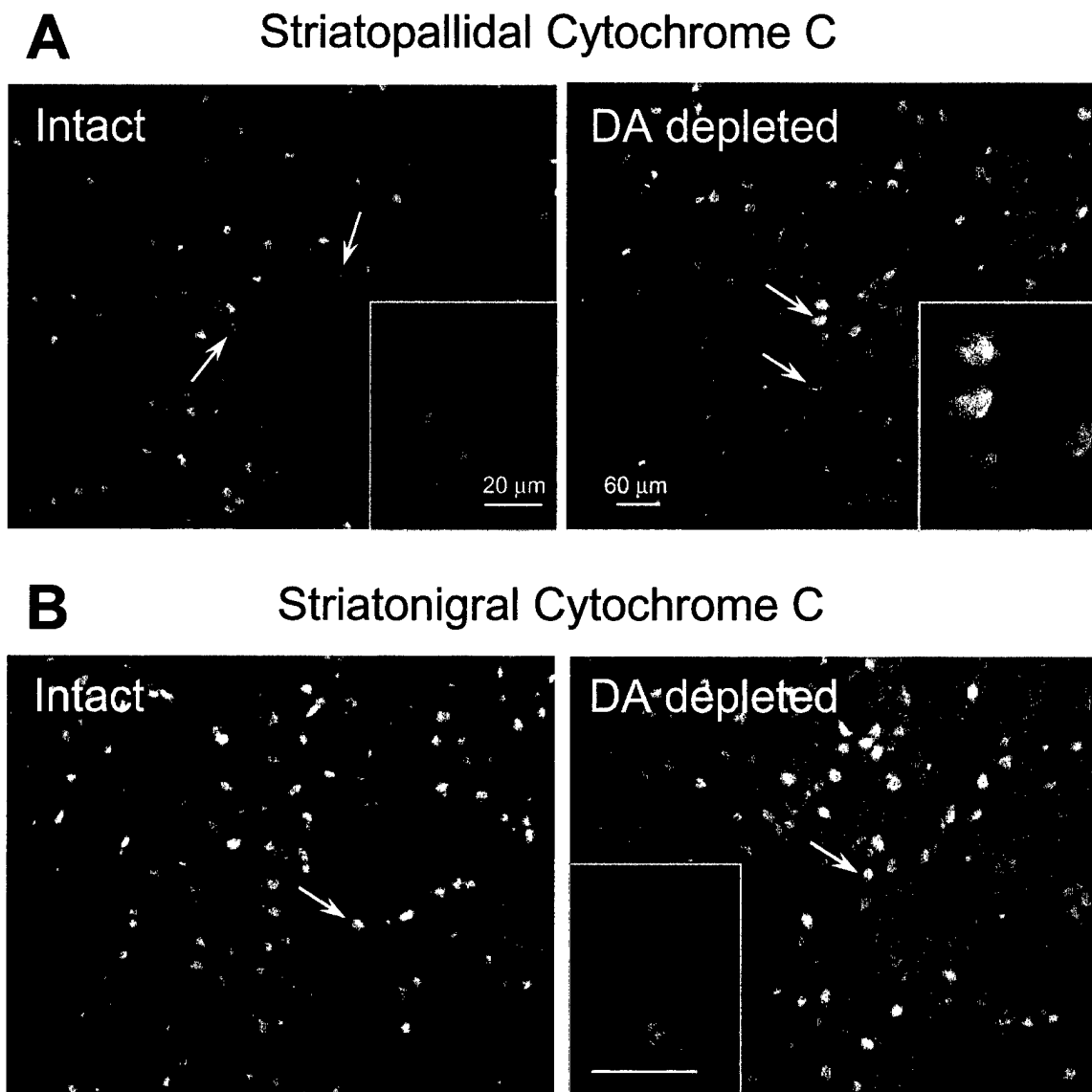

B   Striatonigral Cytochrome C

Figure 6. Cytochrome C staining (red signal) in neuropeptide-identified (green label) striatal projections was evaluated following partial striatal DA depletion. *A,* Labeling of striatopallidal cytochrome C projection neurons (yellow cells, arrows) was enhanced significantly within the DA depleted striatum. The inset showed the rim of cytoplasmic staining for cytochrome C antisera (red signal) in enkephalin-reactive projection neurons (green signal). *B,* Striatonigral cytochrome C colocalization (yellow somata, arrows) was equivalent between the intact and DA depleted striata. Inset demonstrated the thin rim of cytoplasmic cytochrome C (red label) staining visible in typical medium-sized substance P reactive neurons (green signal). Calibration bars are equivalent at 60 μm for lower magnification panels; insets are equivalent at 20 μm.
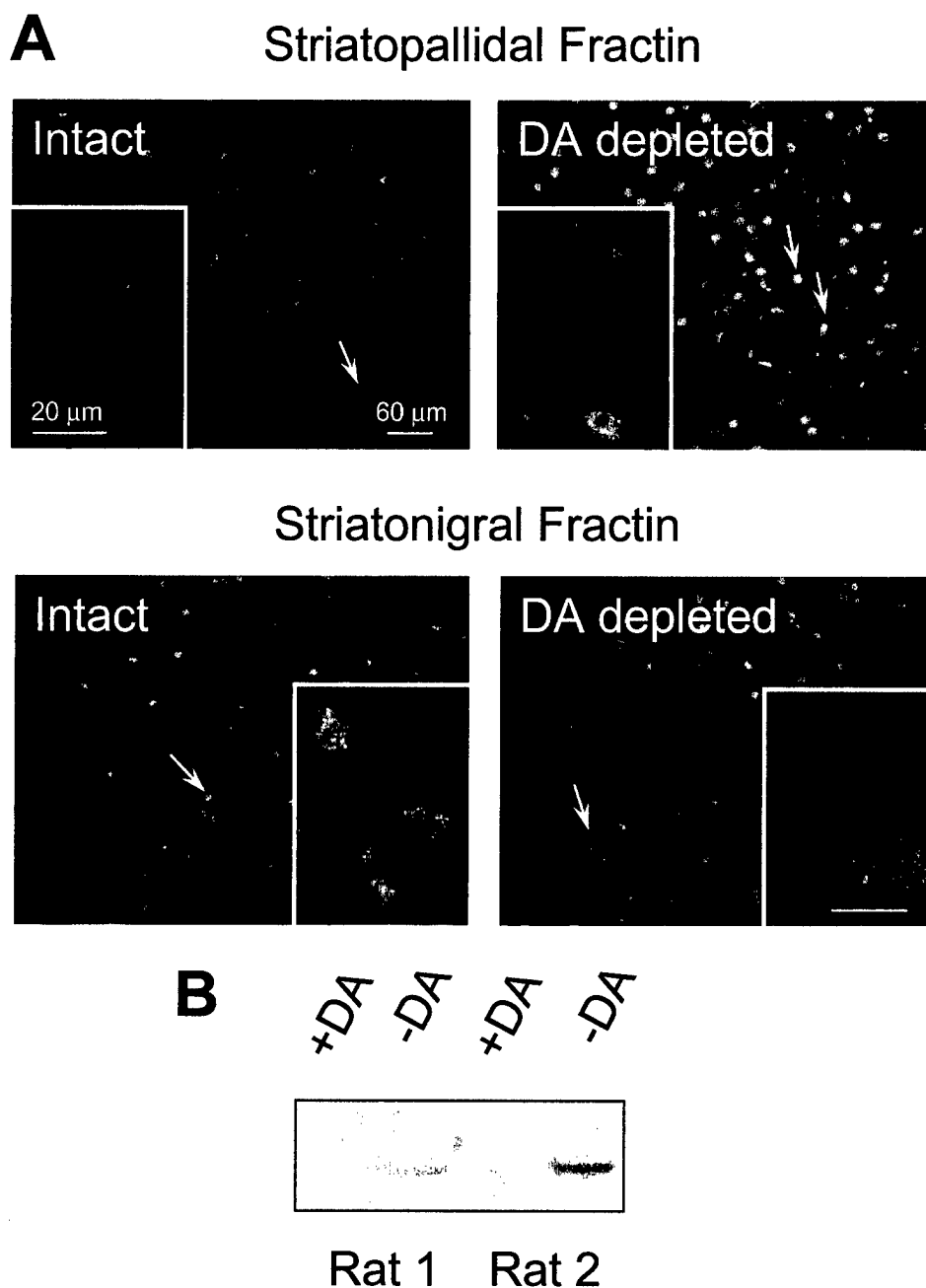
Figure 7

A Striatopallidal Fractin

Striatonigral Fractin

Figure 7. *A*, Striatonigral and striatopallidal (green signals) fractin (red label) co-expression was determined after 6-OHDA. Fractin was distributed in medium diameter neurons of both projection pathways and in the neuropil. After partial DA depletion, fractin fluorescence levels were increased significantly in the striatopallidal system. Insets showed heterogeneous staining for fractin in the cytoplasm of the projection neurons. Calibration bars apply to lower magnification panels at 60 μm, and all insets are equivalent at 20 μm. *B*, Western analysis of whole striatal homogenates from two different rats was assessed using the fractin antisera employed in the morphological studies in *A* and showed +42% elevation of fractin in the DA depleted side contrasted to the intact striatum, $p < 0.05$.